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LANKESTER, E. R., 1904. Quart. J. micr. Sci., **48**, 165.

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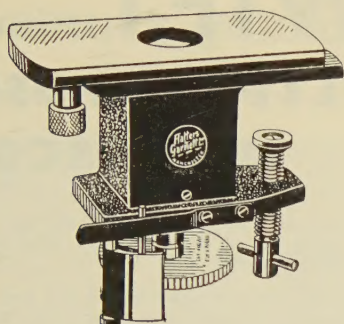
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[1 *front*]

[2]

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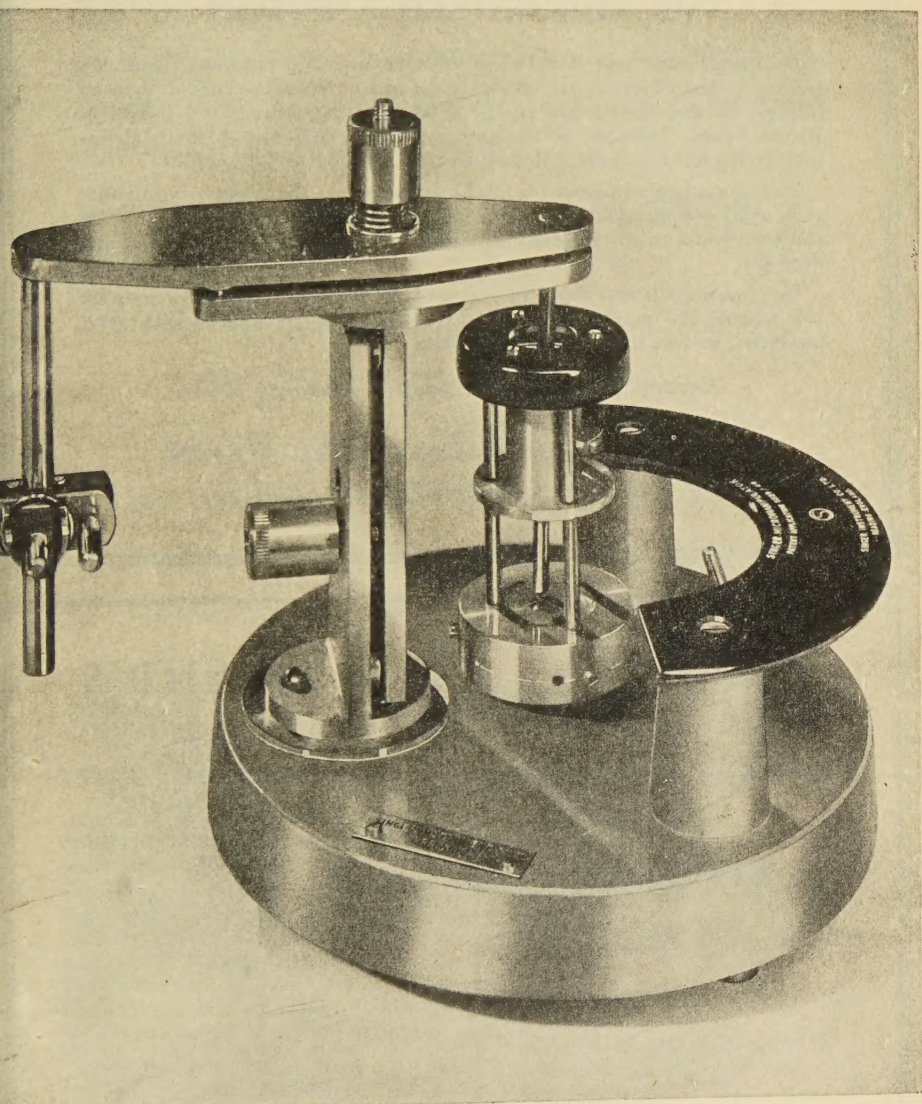
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The Surface of the Sea-urchin Egg

By LORD ROTHSCHILD

(From the Department of Zoology, University of Cambridge, and the Marine Station, Millport)

With one plate (fig. 1)

SUMMARY

Unfertilized eggs of the sea-urchin *Echinus esculentus* were fixed in potassium permanganate, sectioned, and examined under the electron microscope. A membrane consisting of an outer electron-opaque layer 84 Å wide, an electron-transparent layer 57 Å wide, and an inner electron-opaque layer 66 Å wide was observed at the egg surface. No other membrane or electron-opaque layer could be found anywhere within 4 μ of the egg surface.

INTRODUCTION

A PLASMA membrane is often assumed to exist at the surface of cells and to act as an active or passive barrier to the passage of certain substances between the inside of the cell and the external environment. Evidence for the existence of the plasma membrane has been obtained by studying the chemical and electrical properties of cells. The electrical resistance of the surface of a nerve-fibre, for example, is some 100,000,000 times greater than that of the cytoplasm or axoplasm. Moreover, all cell surfaces so far examined have been found to have an electrical capacitance of about 1 μF/cm^2, while no capacitive elements of this size exist in the cytoplasm.

The electrical and chemical evidence suggests that the cell surface or plasma membrane consists of a lipid or lipo-protein layer, 30–100 Å thick, with a dielectric constant of 3–6 (Huxley and Stämpfli, 1949; Fernández-Morán, 1950; Hodgkin, 1951; Davson and Danielli, 1943).

There was no direct evidence for the existence of the plasma membrane until the thin-section electron micrograph technique was developed. Since then, electron-dense layers of about the right thickness have been found at the surfaces of Schwann cells and axons in peripheral nerve-fibres (Robertson, 1957). A similar membrane has also been seen in muscle, liver, skin, kidney, pancreas, endothelial, and endoneurial cells. These cells are all bounded by a 'double' membrane about 75 Å thick (Robertson, personal communication).

The surface or plasma membrane of the sea-urchin egg has usually been assumed to be composed of lipid or lipo-protein; but in 1954, Parpart and Laris produced indirect evidence that the plasma membrane of this egg was not at the surface, but between 1 and 2 μ within, at the inner margin of what is sometimes called the gel-cortex. Parpart and Laris's evidence was twofold. First, they observed that the cortical granules in the unfertilized egg of *Arbacia punctulata* swelled and exploded a few seconds after the eggs were exposed

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2421.1   B

to isosmotic solutions of erythritol and glycerol, though the eggs are relatively impermeable to these substances and do not increase in volume in their presence. Secondly, Parpart and Laris observed under the television microscope that the cortical granules ceased to refract light when the eggs were exposed to isosmotic sucrose solutions and that this could be reversed by replacing the eggs in sea-water. This implies that the sucrose solution came into contact with the granules and had a similar refractive index to them, though sucrose does not penetrate sea-urchin eggs.

Though Parpart and Laris's experiments have only been published as a brief note, it is difficult to conceive of any interpretation of their results other than their own, i.e. that the plasma membrane is beneath the gel-cortex and not at the egg surface. Experiments have therefore been undertaken in the hope of identifying the plasma membrane at the inner surface of the gel-cortex and to re-examine what membrane or membranes are present at the egg surface.

MATERIAL AND METHODS

Unfertilized eggs of *E. esculentus* were fixed in 1% OsO_4, after which they were washed with sea-water. Other unfertilized eggs of the same species were put into 0.6% $KMnO_4$ in sea-water at $0°$ C for 3 h, after which they were washed three times in ice-cold 25% alcohol. Eggs fixed in both ways were dehydrated and embedded in 85% butyl / 15% methyl methacrylate. The plastic was polymerized in the usual way. Ultra-thin sections were then cut.

RESULTS AND DISCUSSION

Fig. 1, A, B shows the surface of the unfertilized sea-urchin egg after $KMnO_4$ fixation. There is a clearly defined double membrane at the egg surface. The average thickness of the outer element of this membrane (measured on enlarged positives of the plate from which A was derived) is 84 (± 2) Å, of the inner element 66 (± 3) Å, and of the space between the two elements 67 (± 3) Å. (The figures within brackets are standard errors of the means.) In spite of the difference in their thickness, the two elements are so similar and the space between them so constant in width, that it seems reasonable to assume that they constitute one membrane and not two separate ones. Trypsin, which inhibits the elevation of the fertilization membrane, may not interfere with both elements. This may explain the fact that the surfaces of trypsin-treated unfertilized sea-urchin eggs do not seem to be different from untreated ones (Mitchison, 1956; unpublished observations of the author). The double membrane, or part of it, is probably the vitelline membrane, the precursor of the fertilization membrane.

OsO_4-fixed eggs only have one osmiophil or electron-opaque layer, 100 Å wide, at their surface (Afzelius, 1956; Rothschild, 1957), though Afzelius

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FIG. 1 (plate). A, part of the surface of an unfertilized sea-urchin egg (*E. esculentus*), fixed in $KMnO_4$. Cortical granules and a double membrane at the egg surface are visible. $\times 7 \times 10^4$.
    B, part of the membrane of the egg surface. $\times 1.4 \times 10^6$.
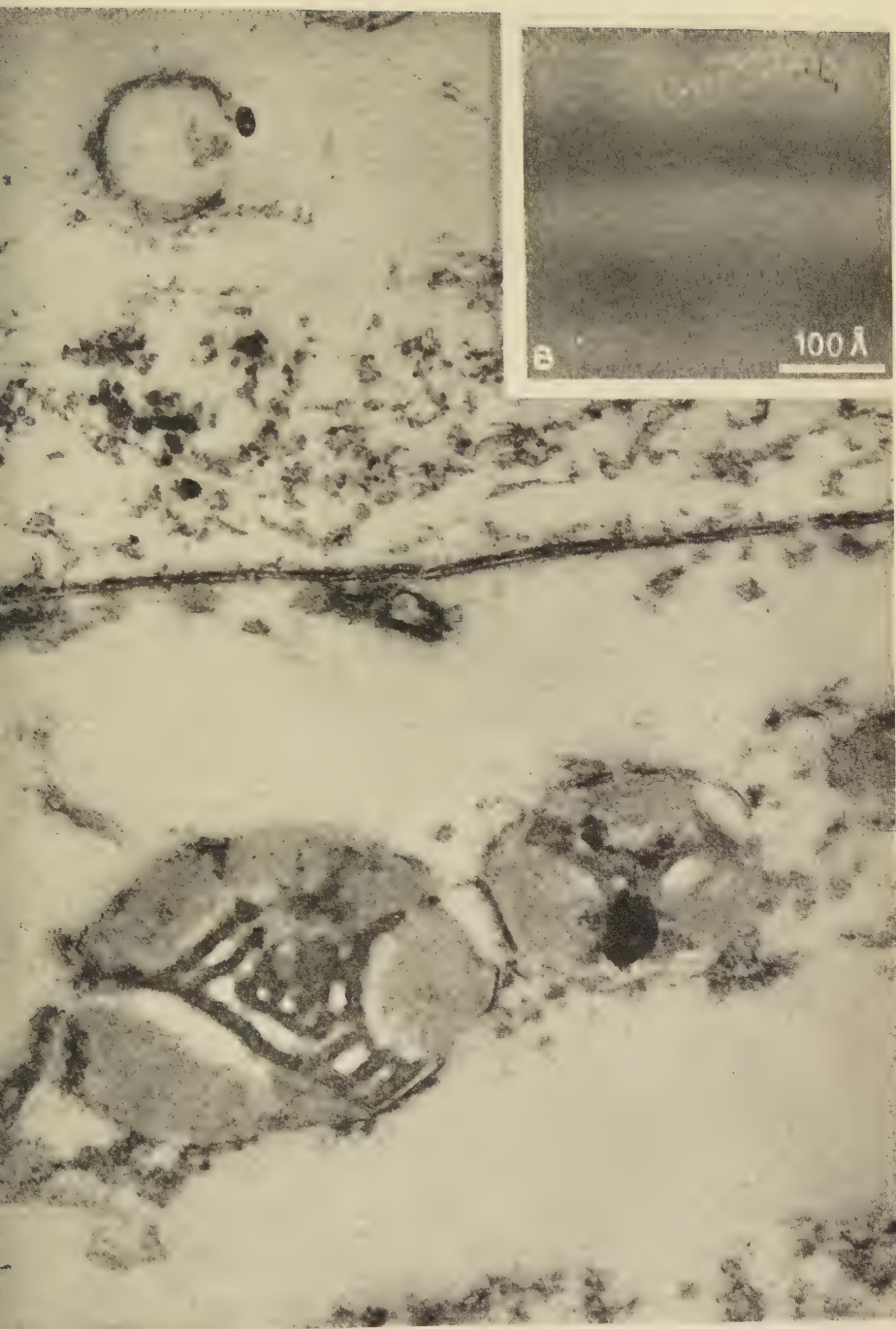
Fig. 1

LORD ROTHSCHILD

says that this membrane sometimes seems to be double. The angle at which the sections are cut will, of course, affect the appearance of the membrane under the electron microscope.

Despite a careful search, no sign of another membrane, between o and 4μ in from the surface of the unfertilized sea-urchin egg, has so far been found, either in OsO_4- or $KMnO_4$-fixed eggs.

In a previous paper (Rothschild, 1957) Runnström was said to have published a diagram of an unfertilized sea-urchin egg in which the plasma membrane was situated under the layer of cortical granules, in 1949, while in 1946 Runnström, Monné, and Wicklund stated that the plasma membrane was outside the cortical granules in the unfertilized sea-urchin egg. Professor Runnström has drawn my attention to the fact that the diagram in the first of these two papers was not of an unfertilized sea-urchin egg but of a just-fertilized one in which the cortical granules had already passed through the plasma membrane. When pointing out this error, Professor Runnström told me he had always been of the opinion that the plasma membrane was outside the layer of cortical granules in the unfertilized sea-urchin egg. I apologize for having unintentionally misrepresented him.

REFERENCES

Afzelius, B. A., 1956. Exp. cell Res., **10**, 257.
Davson, H., and Danielli, J. F., 1943. *The permeability of natural membranes.* Cambridge (University Press).
Fernández-Morán, H., 1950. Exp. cell Res., **1**, 309.
Hodgkin, A. L., 1951. Biol. Rev., **26**, 339.
Huxley, A. F., and Stämpfli, R., 1949. J. Physiol., **108**, 315.
Mitchison, J. M., 1956. Quart. J. micr. Sci., **97**, 109.
Parpart, K., and Laris, P. C., 1954. Biol. Bull., **107**, 301.
Robertson, J. D., 1957. J. biophys. biochem. Cytol., **3**, 1043–8.
Rothschild, Lord. 1957. Ibid., **3**, 103.
Runnström, J., 1949. Adv. Enzymol., **9**, 241.
—— Monné, L., and Wicklund, E., 1946. J. colloid Sci., **1**, 421.

Reactions between Egg-shell Matrix and Metallic Cations

By K. SIMKISS *and* C. TYLER

(*From the Department of Agricultural Chemistry, The University, Reading*)

SUMMARY

The interaction between egg-shell matrix and various metal ions has been studied by considering the effect of these ions on the staining of the matrix by toluidine blue. The validity of the procedure and the influence of pH and various anions were tested. The main hypothesis to emerge was that the matrix acts as a chelating agent. Curves obtained when quantities of sodium hydroxide added to matrix were plotted against pH values were found to vary in position according to the absence or presence of cations and with different cations. This also supports the hypothesis that the matrix acts as a chelating agent. The significance of this in relation to the deposition of calcium carbonate as egg-shell is briefly discussed.

INTRODUCTION

RUBEN and Howard (1950) found that acid mucopolysaccharides occurred at most sites of calcification in the body, whilst Sobel and Burger (1954) have shown that calcium ions can compete with toluidine blue to combine with the organic matrix of rachitic bone. The latter workers then suggested that in the rachitic bone the acid mucopolysaccharide, chondroitin sulphuric acid, was probably the substance for which calcium and toluidine blue were competing. Experiments with other ions led Sobel and Hanok (1952) to state that it was the ionic radius of the various metal ions which caused variations in the degree of inhibition of calcification of the rachitic bone matrix.

Levene and Schubert (1952) approached the problem differently by making a spectrophotometric study of the metachromatism of thiazine dyes produced by chondroitin sulphate. They found that additions of neutral salts of calcium at concentrations of 10^{-3} to 10^{-1} M gave greater losses of metachromatism than did neutral potassium salts.

The organic matrix of hen egg-shell, prepared by the decalcification of egg-shells with ethylenediamine tetra-acetate (EDTA), showed similar properties to rachitic bone, in that calcium ions compete with toluidine blue to combine with it (Simkiss and Tyler, 1957). It was, therefore, decided to investigate the matter further.

VALIDITY OF THE METHOD

Simkiss and Tyler (1957) used EDTA to decalcify egg-shells, and prepared paraffin sections of formalin-fixed decalcified material on slides in the normal way. Individual preparations were then placed in a series of solutions of 0.01% toluidine blue with different concentrations of calcium ions, and the

competition between the dye and the cation for the shell matrix was assessed by the intensity of staining.

Before making more extensive use of this method, it was necessary to test the assumption that dye and cation do compete for the shell matrix.

(1) There is the possibility of a reaction between dye and cation. The results obtained with calcium and described in the previous paper might be explained on the grounds that the metallic ions combined with the toluidine blue to produce a non-staining substance and that the matrix itself took no part in the reaction. An experiment was therefore designed to make sure that this was not the case.

Four serial sections of the egg-shell matrix were prepared as above. The first was placed in toluidine blue (0·01%) to show that it stained metachromatically, the second and third were placed in a solution of 250 m. eq. per litre ferric ions (as $Fe_2(SO_4)_3$) for 1 h. They were then removed and drained, and whilst the second was put into a 10% solution of the trisodium salt of EDTA to remove the ferric ions, the third was left in glass-distilled water. After 2 h the slide in the EDTA was removed, washed, and left for 1 h in glass-distilled water to wash out any remaining EDTA from the section. Both slides were then placed in 0·01% toluidine blue solution. The slide which had been treated with EDTA stained metachromatically after 5 min, the other showed no staining even after 12 h. The fourth slide was placed in the stain with the ferric ion treated sections. This slide stained metachromatically, showing that the stain itself had not been destroyed.

From this experiment it may be argued that, since the ferric ions and the stain never come together in the free state, the loss of staining must be caused by a combination of the cation and the egg-shell matrix and not by a direct interaction between the ferric ion and the stain. Furthermore, the removal of the ferric ions by means of EDTA regenerates the staining property of the shell matrix.

It may still be suggested that the slide treated with ferric ions fails to stain because the ions come off the section and react with the dye to give a colourless compound, but the result with the fourth slide shows that this cannot be so because the slide stains even though placed in the same solution as that occupied by the slide containing ferric ions. In any event, there is such an excess of toluidine blue over ferric ions that the dye could not all be changed to a non-staining substance. It seems, therefore, that the method is valid in that a combination does actually occur between the shell matrix and cations.

(2) There might be residual EDTA in the sections. EDTA may have combined with the shell matrix during decalcification and therefore it might be the EDTA and not the actual shell matrix which is combining with the cations. To clarify this point, egg-shells were dissolved in hydrochloric acid and washed, and the residue was then dried on to slides. These pieces of shell stained metachromatically with toluidine blue solution and staining could be prevented by using solutions of calcium ions just as it could when EDTA had been used (Simkiss and Tyler, 1957). Thus, acid-decalcified shells showed

the same properties as ones treated with EDTA, and it may, therefore, be concluded that any reaction of the shell matrix with cations is due to the shell matrix itself and not to any EDTA attached to it.

We may thus conclude that the cations do compete with toluidine blue for combination with the matrix. The next stage was to use the method for assessing the competitive value of different cations.

EFFECT OF DIFFERENT CATIONS

A series of experiments was set up for each solution, with increasing concentrations of the metal ion in 0·01% toluidine blue solution. Sections were left in these solutions for at least 30 min to reach equilibrium and then the degree of metachromatic staining of the matrix was scored with values between O and + + + + as judged by eye. The concentration of cation which produced a marked fall or complete elimination of the staining in the section will be referred to as the 'elimination concentration'.

TABLE I

Elimination concentrations (m. eq. per litre) for cations used in conjunction with different anions

Salt	Elimination concentration	pH at elimination concentration
$FeCl_3$	1	3·60
$Fe_2(SO_4)_3$	1	—
$AlCl_3$	1	4·34
$Al_2(SO_4)_3$	1	—
$BeCl_2$*	5	—
$CuCl_2$	5	4·64
$CuSO_4$	4	—
$NiCl_2$	70	5·06
$Ni(NO_3)_2$	70	—
$ZnCl_2$	100	5·50
$Zn(NO_3)_2$	100	—
$FeCl_2$*	—	3·60
$FeSO_4$	100	—
$MnCl_2$	160	5·00
$MnSO_4$	160	—
$CaCl_2$	180	5·10
$SrCl_2$	220	4·80
$LiCl$	325	5·84
$NaCl$	400	5·50

* Impure substance.

Water of crystallization not included in formulae.

The results are shown in table 1. Each cation was tested along with a chloride ion and then various other anions were used with one or other of the cations. From the table it will be seen that the sulphates and nitrates behaved like the chlorides, the only exception being with ferrous salts. With ferrous sulphate the elimination concentration occurred at 100 m. eq. per litre but with ferrous chloride no staining was obtained with toluidine blue even with

only 50 m. eq. per litre ferrous ions. It may be that this discrepancy is caused by impurities in the ferrous chloride, for it was not possible to obtain this material at analytical reagent standard. Furthermore, this substance readily oxidizes to the ferric state.

Effect of pH

No attempt was made to buffer the solutions, since this would lead to complications by adding other cations or competing anions. Measurements of pH were, therefore, made at the elimination concentration. Table 1 shows that the range of pH was from 3·6–5·8 with most of the results between 4·6 and 5·6. Further experiments were carried out to measure the effect these pH differences were having on the staining of the matrix. Solutions of cupric chloride (pH 4·6), calcium chloride (pH 5·1), and sodium chloride (pH 5·5) were brought to a pH of about 3·6 by adding dilute hydrochloric acid, and the results of staining were compared with the untreated solutions as shown in Table 2. Increased acidity results in a fall in staining intensity. The elimination concentration is not altered, however, by this fainter staining. In those

TABLE 2

Effect of pH on staining (O to ++++) with toluidine blue in the presence of various concentrations of cation

Concentration of solution (m. eq. per litre)	Sodium		Calcium		Cupric	
	pH 5·50	*pH* 3·60	*pH* 5·10	*pH* 3·60	*pH* 4·64	*pH* 3·60
500	trace	trace	O	O	O	O
400	++	+	O	O	O	O
250	++++	++	O	O	O	O
200	++++	++	+	trace	O	O
150	++++	—	++	+	O	O
100	++++	—	++++	++	O	O
10	++++	—	++++	—	trace	trace
5	++++	—	++++	—	++	+
0	++++	++	++++	++	++++	++

cases where the elimination concentration is shown by a marked fall in staining, the lower general level of staining does not affect the point where the marked fall occurs. Where complete elimination of staining occurs, the additional competition of the hydrogen ions may affect the result slightly. However, this is only likely to affect cupric, beryllium, aluminium, and ferric ions, which, in any event, give a very small value for the elimination concentration. They are also the cations which are most likely to produce the most stable compounds with the matrix and, therefore, would be the least affected by the slight increase in competition due to hydrogen ions. The results with cupric ions at pH 3·6 illustrate this experimentally. From this it is concluded that over the range of values used, the pH affected the intensity of staining but not the elimination concentration.

ELIMINATION CONCENTRATION AND STABILITY

Ignoring the unreliable ferrous chloride result, it is possible to put the cations in a series of decreasing elimination concentrations as follows:

$$Na^+ \; Li^+ \; Sr^{++} \; Ca^{++} \; Mn^{++} \; (Fe^{++}) \; Zn^{++} \; Ni^{++} \; Cu^{++} \; Be^{++} \; Al^{+++} \; Fe^{+++}$$
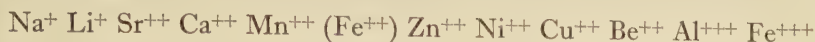
It seems likely that the elimination concentration for staining gives some measure of the relative affinity of the shell matrix for different ions, a high value indicating little affinity. As the combination of the cation and the shell matrix is presumably in a state of equilibrium, an experiment which measures the stability of the metal-matrix compound should give a similar series to that for the elimination concentration. The relationship of the two may be expressed as follows:

$$\underset{\text{(stainable)}}{M+S} \underset{\text{stability}}{\overset{\text{elimination concentration}}{\rightleftharpoons}} \underset{\text{(unstainable)}}{MS},$$
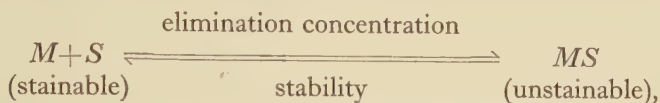
where M is the cation, S is the shell matrix, and MS is the combination of the two.

In measuring elimination concentration we are, presumably, obtaining some measure of the concentration of M which is necessary to displace the equilibrium so that MS (unstainable) forms at the expense of S (stainable). This presumption can be tested by the converse experiment of measuring the stability of MS when conditions are created for its breakdown.

In an attempt to measure stability the following experiment was tried. Sections of shell matrix were placed in solutions containing 250 m. eq. per litre of the various cations. After 12 h, when it was assumed that the shell matrix and cation would have combined to form the non-staining compound, the slides were drained and transferred to 0·01% toluidine blue solution. They were then observed at regular intervals until metachromatic staining was obtained, i.e. until the reaction produced sufficient of the compound S to be detected by staining. The time required gave a measure of the stability of MS.

The results did not enable a series to be established but the general picture strongly supported the evidence based on elimination concentrations. Ferric and aluminium ions gave very stable compounds between cation and shell matrix, for even after 4 days there was no staining with the slides treated with ferric ion and it was only very slight with aluminium. The slide treated with beryllium was stable for about 22 h, cupric ions gave about 5 min stability, and the other cations of Table 1 produced compounds of less than 3 min stability. Even at 500 m. eq. per litre lithium and sodium showed very low stability.

As will be explained in the discussion, this evidence suggested that the shell matrix might contain a chelating agent and therefore it was decided to carry out titration experiments in an effort to obtain more evidence of chelation.

Before leaving this aspect of the subject, it should be stressed that the descriptions of staining with toluidine blue refer to the general state of the section, but that there is considerable evidence to suggest that different layers of the shell stain with a somewhat different intensity.

<div align="center">TITRATION EXPERIMENTS</div>

Martell and Calvin (1952) have pointed out that shifts in the titration curves for compounds in the presence and absence of cations are a quick and simple method of testing for chelation, because the process of chelation results in the displacement of protons.

Twelve to fifteen hen egg shells were carefully washed in warm water and then placed in 2N hydrochloric acid for a few seconds. The acid quickly seeped through the organic cuticle and membranes and began to attack the true shell. The cuticle and membranes were thus loosened and the cuticle could then be easily wiped off and the membrane could be peeled off. These shells were then broken into small pieces and dissolved in about 3 l. of N hydrochloric acid, containing 5% formalin to prevent any solution of the organic matter. When all the calcium carbonate of the shell had dissolved, the suspension of organic matter was centrifuged at 2000 r.p.m. for 30 min and the supernatant acid poured away. The gel was then broken up with a jet of water and re-centrifuged, after which it was taken up as a suspension in approximately 50 ml N/100 hydrochloric acid.

It was impossible to obtain exactly the same amount of matrix in each aliquot of the suspension, hence there was no need to ensure that the N/100 acid was absolutely accurate. However, for each batch, the titrations gave consistent results.

Twenty or twenty-five ml portions of the matrix suspension, with or without additions of cations, were titrated with N/50 sodium hydroxide solution and pH readings were taken after each addition. Titrations were made on the following suspensions: (*a*) suspension alone, (*b*) suspension plus 0·1 m. eq. copper as cupric chloride ($CuCl_2 . 2H_2O$), (*c*) suspension plus 0·2 m. eq. copper, (*d*) suspension plus 0·2 m. eq. nickel as nickel nitrate ($Ni(NO_3)_2 . 6H_2O$), (*e*) suspension from shells boiled for 14 hours with 10% sodium hydroxide solution (Tyler and Geake, 1953) before preparing the matrix from them, (*f*) alkali treated shell plus 0·1 m. eq. copper, and (*g*) N/100 solution of HCl plus 0·1 m. eq. copper.

Some of the results are shown in fig. 1, but values for additions of alkali below 10 ml have been omitted since they merely represent the neutralization of excess acid and give more or less similar readings for all curves. The suspension of matrix (*a*) and of treated matrix (*e*) give smooth curves but they are in quite different positions. The curve for copper in acid (*g*) shows a step at about pH 6·5, which presumably represents the precipitation of copper as the hydroxide. When the matrix suspension is titrated in the presence of 0·1 m. eq. of copper, the curve (*b*) gives a small step at about pH 6·5 associated with precipitation, but at much lower pH values, starting about pH 3·5, there

is a considerable deviation of the curve away from curve (*a*). The curve for
treated matrix in the presence of copper (*f*) gave the step associated with pre-
cipitation but little or no deviation, for, until pH 5·7 is reached, this curve is
very similar to the curve for treated matrix alone (*e*). Although the curves
(*c*) and (*d*) are not presented, it may be stated that with twice the amount of
copper present, the step related to precipitation is again from pH 6 to 7, but

Fig. 1. Titration curves for shell matrix against N/50 sodium hydroxide.
a, matrix alone; *b*, matrix plus copper; *e*, treated matrix alone; *f*, treated
matrix plus copper; *g*, copper alone. Curves *c* and *d* are omitted to avoid
confusion.

covers a wider range of sodium hydroxide additions; once more, however,
there is the deviation starting at about pH 3. The use of nickel gave a step
probably associated with precipitation, but also a deviation starting at about
pH 4.

These results show that there are steps in the curves when copper and nickel
are present and these are associated with precipitation of the hydroxide. In
addition, the presence of nickel produces a shift in the curve for the matrix
from a normal shell, whilst copper produces an even greater displacement.
The shift in these curves shows a displacement of protons, a result typical of
chelation. This shift does not occur when matrix from an alkali-treated shell
is used, which suggests that the chelating agent in the matrix has been removed
by this treatment.

DISCUSSION

Sobel and Hanok (1952) considered that the organic matrix of rachitic bone combined with various cations according to their ionic radii, but, in the present experiments with decalcified shell matrix, the elimination concentrations and the stability of the metal-matrix compounds do not vary in any direct way either with the ionic radius or, for that matter, with the ionic charge.

Further examination of the results indicates that the series obtained was similar to that for the formation constants of chelation compounds. This
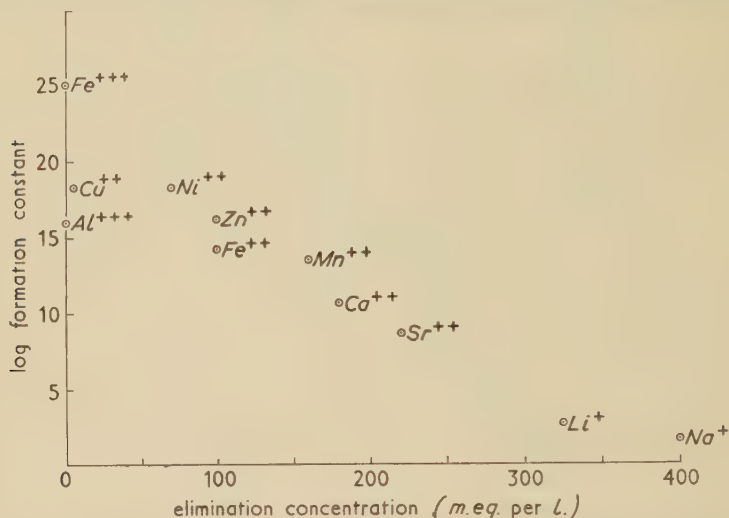
FIG. 2. Graph showing the relationship between the elimination concentration and the logarithm of the formation constant of EDTA chelation compounds for various cations.

relationship is shown in fig. 2, where the elimination concentration has been plotted against the logarithm of the formation constant of EDTA chelation compounds for each cation (Bersworth Chemicals, 1953). Chelation involves a union between the metallic ion and the chelating molecule. The bond arises from dative covalencies, and since in chelation at least two bonds hold the molecule to the metal ion, the structure consists of one or more rings. Different metals have different capacities for co-ordination and there is thus a natural order of metals in all co-ordination problems which is only slightly modified in particular cases by the relative affinity of particular groups for particular metals. In this respect, Irving and Williams (1948) have shown that in the absence of any major spatial limitations in the structure of the molecule, the order of intensity of chelation of metals of the first transition series is independent of the nature of the chelating agent. Thus it would be expected that if there is a chelating agent in the shell matrix, it would not only chelate with metals other than calcium, but would do so in a particular series.

The series of the elimination concentrations for different cations shown here is, in fact, similar to the chelation series of cations, and the variations in stability of the cation-matrix complex (MS) with different cations gives some support

to this parallelism. It is known that the strength of chelation will increase with an increase in ionic charge, but in chelation other factors are also involved which complicate a simple relationship with either ionic charge or radius. On the basis of the type of test described by Martell and Calvin (1952), the titration curves obtained show a displacement of protons and thus support the idea that chelation is occurring.

The elimination concentration and stability experiments therefore suggest chelation and the shifts in the titration curve support this concept. Simkiss and Tyler (1957) have shown that the shell matrix contains an acid mucopoly-saccharide, probably mucoitin sulphuric acid, and it is now suggested that this is probably the substance which is responsible for the chelation. Mucoitin sulphuric acid contains secondary amino, hydroxyl, carboxyl, and sulphate groups, any of which could take part in the formation of chelate rings, but it must be remembered that some of these groups may be bound to the protein in the matrix and hence it is not yet possible to be more specific as to which groups are involved. In addition, the effect of formalin fixation on protein may alter the picture.

The role played by mucoitin sulphuric acid as a chelating agent in the egg-shell may be of considerable importance to the question of calcification, although the experimental evidence suggests that the chelation compound between calcium and shell matrix is not very stable.

In the hen the calcium is only present in a weak solution in the blood; at the very most 40 mg Ca/100 ml serum, i.e. 20 m. eq. per litre. A chelating agent might act by removing calcium from this weak solution with the formation of a calcium-matrix compound, until carbonate ions can rob it of its calcium ions and form calcium carbonate and free matrix. This mechanism would ensure the precipitation in the shell itself of calcium carbonate, intimately fitted into a definite structure with the matrix, whereas the direct interaction of free calcium and carbonate ions does not seem to offer the same opportunity to build up a structure such as is found in egg shells.

REFERENCES

Bersworth Chemical Co., 1953. *The versenes*, 6th edition, p. 7. Massachusetts (Bersworth Chemicals).
Irving, H., and Williams, R. J. P., 1948. Nature, **62**, 746.
Levene, A., and Schubert, M., 1952. J. Am. Chem. Soc., **74**, 91.
Martell, A. E., and Calvin, M., 1952. *Chemistry of the metal chelate compounds*. New York (Prentice-Hall).
Ruben, P. S., and Howard, J. E., 1950. *2nd Conference on Metabolic Inter-relations, 155*. New York (Macy Foundation).
Simkiss, K., and Tyler, C., 1957. Quart. J. micr. Sci., **98**, 19.
Sobel, A. E., and Burger, M., 1954. Proc. Soc. exp. Biol. N.Y., **87**, 7.
—— and Hanok, A., 1952. J. biol. Chem., **197**, 669.
Tyler, C., and Geake, F. H., 1953. J. Sc. Fd. Agric., **4**, 261.

The Spermatid of the House-cricket, *Acheta domesticus*

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With five plates (figs. 2–6)

SUMMARY

1. The spermatid of the house-cricket, *Acheta domesticus*, has been studied by the use of vital dyes, histochemical tests, and electron microscopy.

2. The acroblast consists of an outer and an inner part. The former, which contains lipids and a neutral mucopolysaccharide, is seen in the electron micrographs to consist of parallel lamellae. In section these appear to be arranged in the form of a horse-shoe. The internal part of the acroblast gives feeble reactions for a neutral mucopolysaccharide and for a lipid. In electron micrographs it is seen to contain minute vesicles.

3. The acrosome arises within the acroblast. It gives a strong reaction for neutral mucopolysaccharide. It appears to be devoid of internal structure.

4. The *Nebenkern*, formed by the fusion of all the mitochondria in the young spermatid, exhibits the 'cristae' characteristic of ordinary mitochondria. The reactions of this structure with vital dyes and acid fuchsine also parallel those of the mitochondria. Histochemical tests indicate the presence of a lipid.

5. The periphery of the nucleus of the late spermatid shows a radial structure.

INTRODUCTION

THE spermatogenesis of the house-cricket, *Acheta domesticus* (L.), has been worked out by Nath and Bhimber (1953), who studied living cells by phase-contrast microscopy and also used testes fixed in Lewitsky's and Champy's fluids; paraffin sections were stained in iron haematoxylin.

The cricket is a very convenient animal for studies of the cytoplasmic inclusions in spermatogenesis, because the animal lives and breeds readily in the laboratory, the testes are large and very easy to dissect out, and the cytoplasmic inclusions of the male germ-cells easy to study by light microscopy. We have therefore sought to extend the work of Nath and Bhimber by the use of vital dyes, histochemical tests, and electron microscopy. Our attention has been concentrated mainly on the young spermatid, though we have also made some observations on the later stages, including the spermatozoon.

It is necessary at the outset to review briefly the transformation of the spermatid into the spermatozoon. We have confirmed the main features of Nath and Bhimber's findings.

The young spermatid, just before it starts to elongate, is represented diagrammatically in fig. 1, A. It contains a spherical nucleus, a subspherical *Nebenkern* formed by the fusion of all the mitochondria in the cell, and an

object supposed to be formed by the fusion of most of the lipid droplets or 'Golgi bodies'. This object is called the acroblast, because the acrosome first appears within it.

At a later stage (fig. 1, B), an axial filament extends from the centriole, which later moves to the end of the cell that will become the anterior end of the spermatozoon; the tail grows out; the acrosome appears within the internum of the acroblast; the *Nebenkern* divides, and the two halves extend along the tail.
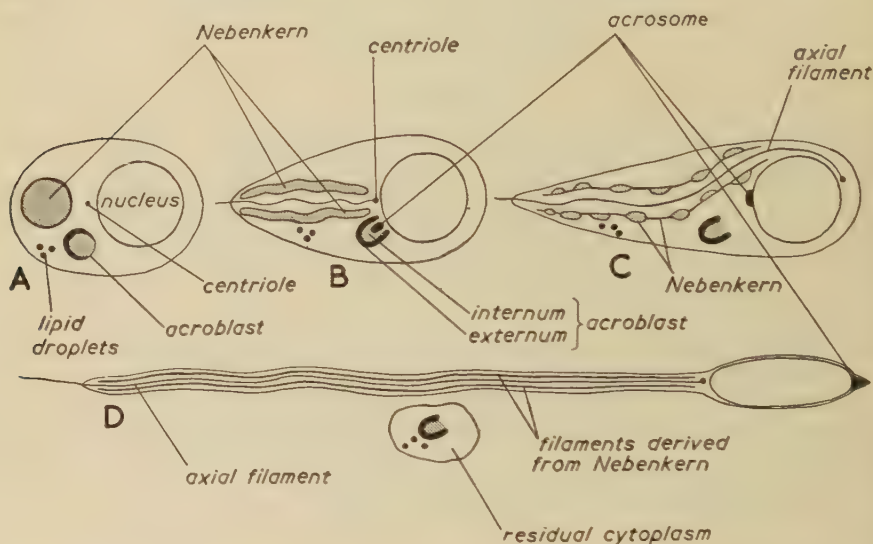
FIG. 1. Diagrams of four stages in the development of the spermatid of the house-cricket.

The acrosome now becomes attached to the nucleus, and the acroblast moves away from it (fig. 1, C); the halves of the *Nebenkern* spin out into threads lying beside the axial filament; these are swollen here and there into blebs.

Later the acrosome appears at the anterior end (fig. 1, D); probably this change in position is due to rotation of the nucleus through 180°. The residual cytoplasm is thrown off. It contains those lipid granules that did not take part in the formation of the acroblast, and also of the acrosome itself.

The parts of the cell are now arranged as they will be in the spermatozoon, and it only remains for the acrosome, nucleus, and tail to lengthen and become attenuated.

FIG. 2 (plate). A, spermatid, early stage. Mitochondria (*m*); nuclei (*n*) with nuclear membrane (*nm*), and electron-dense object (*ni*) inside the nucleus.

B, spermatid, more advanced stage. Laminae (*mb*) in the ground cytoplasm; acroblast (*aa*) showing laminated structure of the externum and associated vesicles, also vesicles in the internum; acrosome (*a*); *Nebenkern* (*nk*) with double membranes (*mm*); axial filament (*f*). surrounded by a membrane; nuclei (*n*).
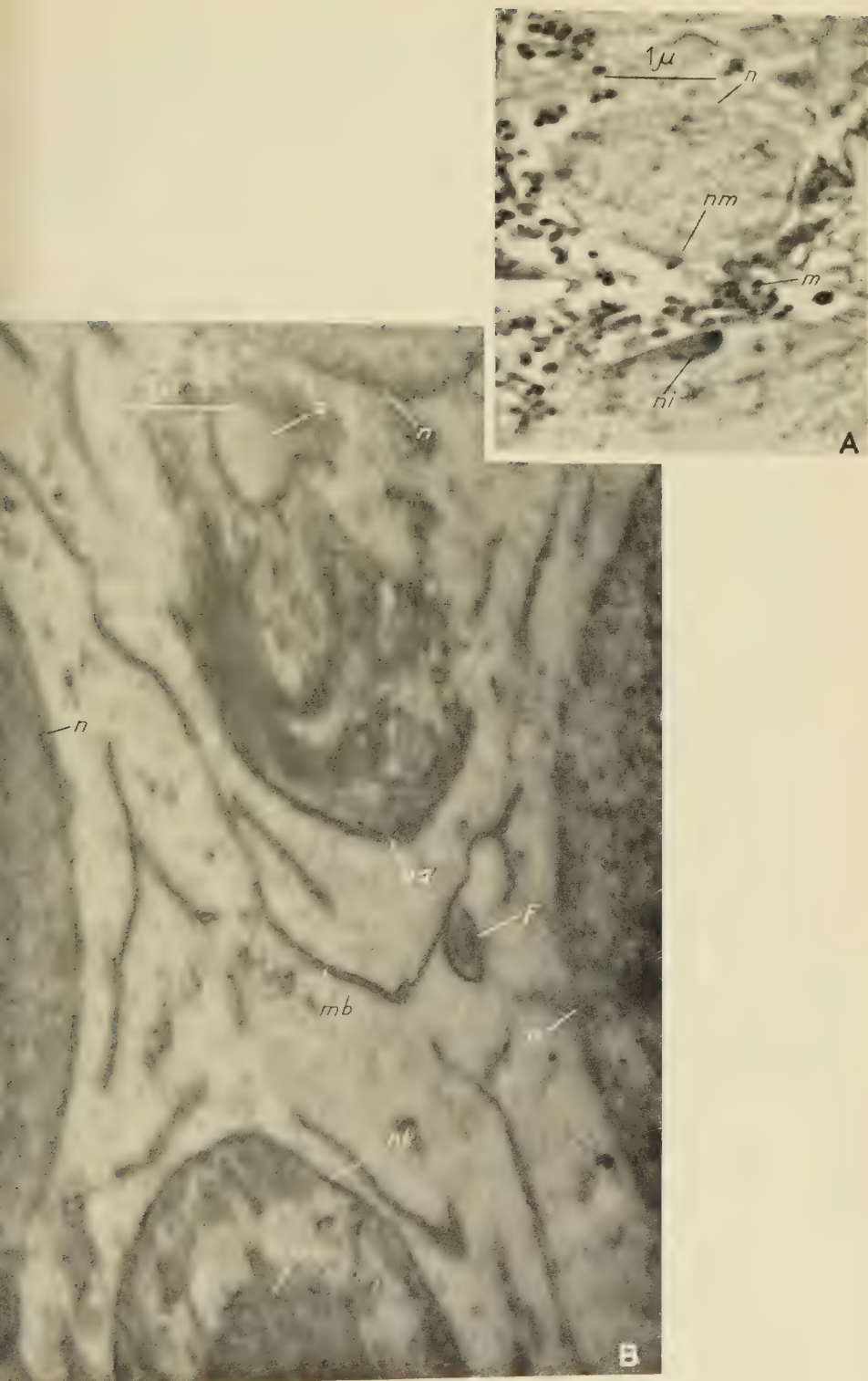
Fig. 2

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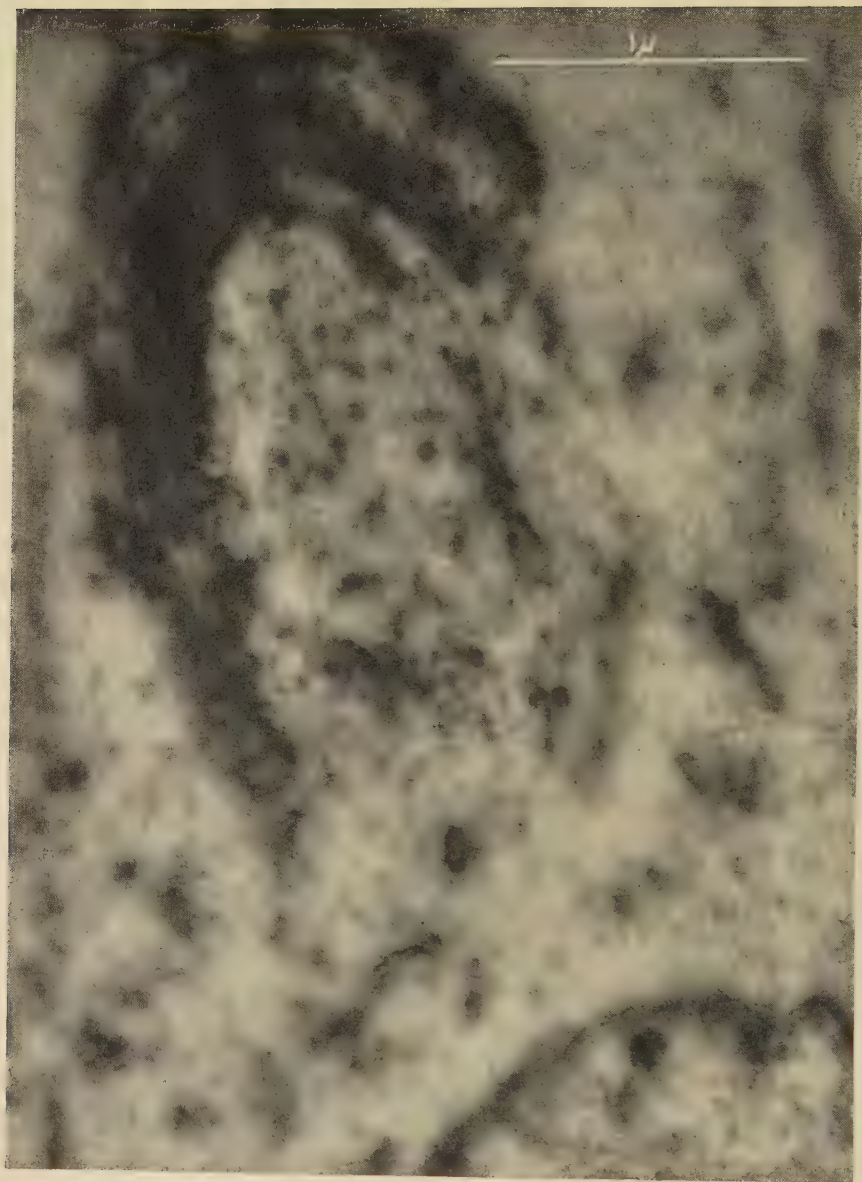
FIG. 3

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MATERIALS and METHODS

The crickets were cultured by the method of Jordan and Baker (1956). For the study of the living cells, the testes were teased in this solution of insect saline:

Sodium chloride, 10% . . . . 10 ml
Distilled water . . . . 90 ml
Calcium chloride, anhydrous, 10% . . . . 0·2 ml

The following vital dyes were used: neutral red, Janus green and black, brilliant cresyl blue, thionine, methylene blue, and dahlia violet. All, except methylene blue (0·04%) and brilliant cresyl blue (0·01%), were dissolved in the saline solution at 0·02%. The cells were dyed for about $\frac{1}{2}$ to 1 hour in covered watch-glasses and then placed on slides, covered, and at once examined.

The mitochondrial technique of Metzner (Metzner and Krause, 1928) and the 'Golgi techniques' of Aoyama (1929), Kolatchev (1916), and Weigl (1910) were used.

Details of the above tests and the histochemical tests are given in the appendix (p. 22).

For electron microscopy the testes of the cricket were rapidly dissected out. Small blocks from the anterior region were fixed in 1% osmium tetroxide dissolved in the insect saline mentioned above. The tissue was dehydrated by standard methods, sectioned at about 25 mμ with a Porter–Blum–Servall microtome. Micrographs, taken in a Siemens Elmiskop I at instrumental magnifications 2,600 and 8,000, were enlarged photographically.

RESULTS

Ground cytoplasm

In the electron micrographs this appears to have the usual characters (figs. 2, A, B; 3; 4, A–C; 5, A, B). The background is spotted with small granules. Laminae with double membranes appear to be dispersed at random throughout the cytoplasm, without orientation. It is clear that the laminae must have considerable extension, because great lengths of them appear in all sections. The laminae show a general resemblance to the endoplasmic reticulum of Palade (1956), but no granules have been noticed on the surfaces of the laminae. The laminae are about 30 mμ thick. They never seem to swell into vesicles.

Cell membranes have not been distinctly seen. The possibility exists that at certain stages the spermatids form a syncytium, limited only by the cyst wall. Later, however, the spermatids are obviously separate from one another and from the wall (figs. 5, C, D; 6, A, B).

FIG. 3 (plate). Acroblast before formation of acrosome, showing laminated structure of externum.

Acroblast

The acroblast consists of two regions: an outer laminated part, the externum, which appears horseshoe-shaped in section, and an inner area, the internum, which is apparently structureless.

The externum of the acroblast was coloured by the vital dyes dahlia and Janus black. The externum was coloured red by Metzner's method for mitochondria, and was blackened by osmium in the Baker (Hermann–Kopsch) and Weigl (Mann–Kopsch) techniques, and by silver in Aoyama's method. All these techniques left the internum negative or less positive than the externum.

Of all the histochemical tests tried, only those for lipids and polysaccharides gave positive results. The externum was blackened both by Sudan black and by acid haematein, but it was found not possible to analyse further the lipid constituent responsible for these reactions. Both the internum (feebly) and the externum (strongly) gave positive reactions to the periodic acid / Schiff (PAS) test, especially after fixation in Bouin's fluid. The reaction was even more positive after the sections had been treated by salivary amylase. No part of the acroblast was spontaneously metachromatic, but treatment on the slide with sulphuric acid (Lison, 1953) resulted in a positive reaction by both internum and externum. These facts indicate the presence of a lipid and may indicate the presence of a neutral polysaccharide, especially in the externum.

The externum and the internum are clearly differentiated in the electron micrographs. The externum appears in the form of parallel laminae with double membranes, mostly in close apposition to one another, all bent in the form of a horseshoe (figs. 2, B; 3). Vesicles are occasionally seen in the externum. These reach a diameter of about 150 mμ. In fig. 2, B they are confined to one side of the organelle. The double membranes of the externum are electron-dense. This may perhaps be due to reduction of osmium tetroxide by unsaturated lipid.

The internum contains numerous, randomly dispersed vesicles, about 30 mμ in diameter. The intervening material is as transparent to electrons as the background of the cytoplasm.

Acrosome

Neutral red was the only vital dye that coloured the acrosome. It was also coloured red by acid fuchsine in Metzner's method. It was negative to all tests for lipids except to the acid haematein technique; it responded positively to the PAS test even after treatment with saliva. It became metachromatic after treatment on the slide with sulphuric acid. Thus the histochemical tests

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Fig. 4 (plate). A, spermatid, more advanced stage. Double laminae (*mb*) in the ground cytoplasm; *Nebenkern* (*nk*) with double membranes (*mm*); nucleus (*n*).

B, spermatid, more advanced stage. *Nebenkern* (*nk*) dividing; double membranes (*mm*); nucleus (*n*) with electron-dense object (*ni*); laminae (*mb*).

C, spermatid, more advanced stage. *Nebenkern* (*nk*) dividing, double membranes still present; nuclei (*n*) with electron-dense object (*ni*).
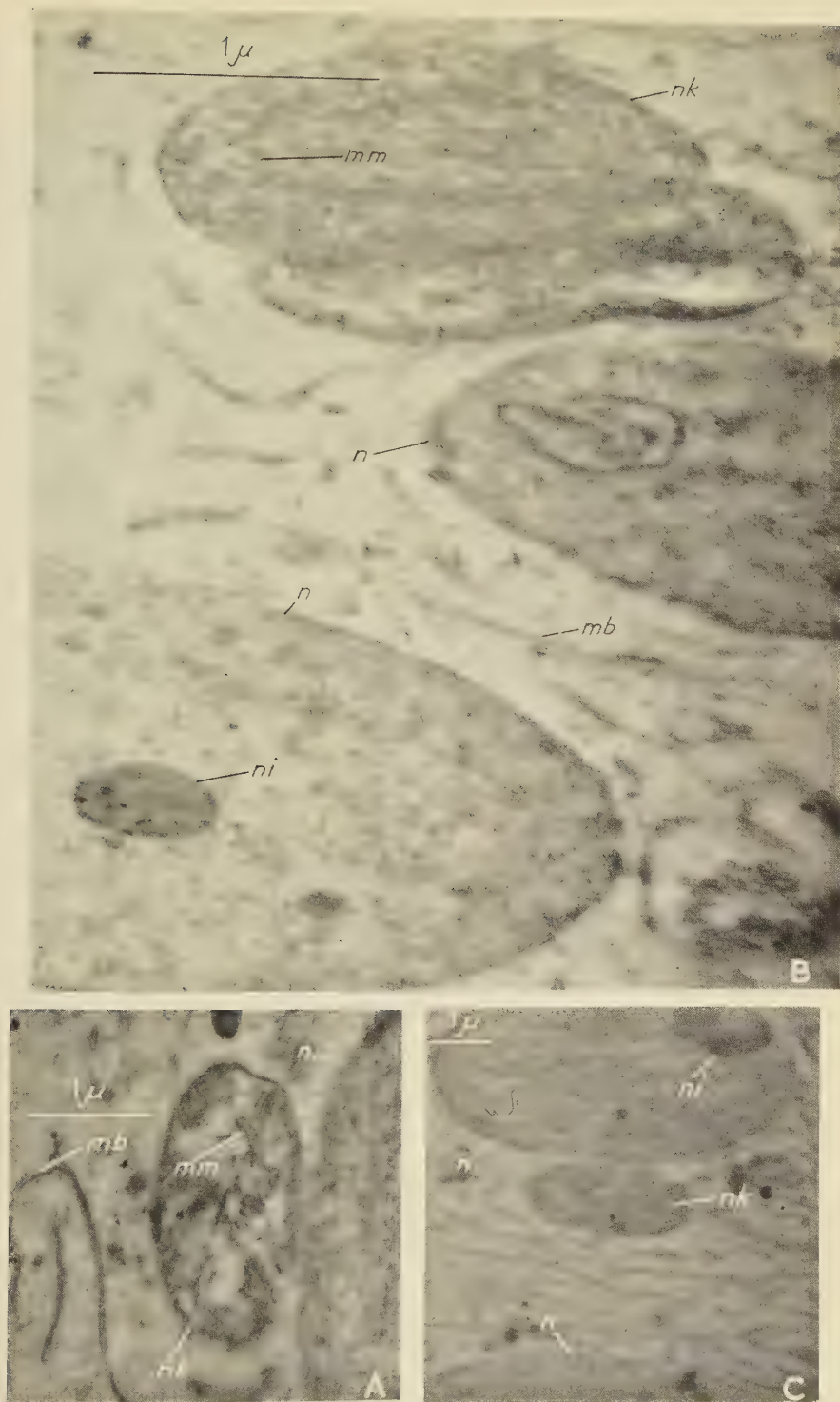
FIG. 4

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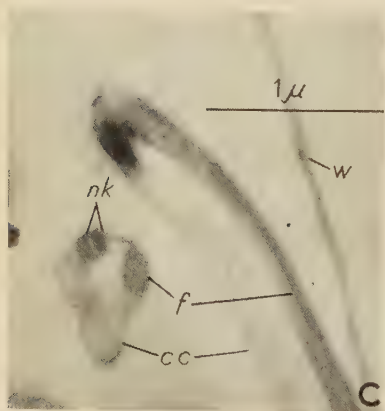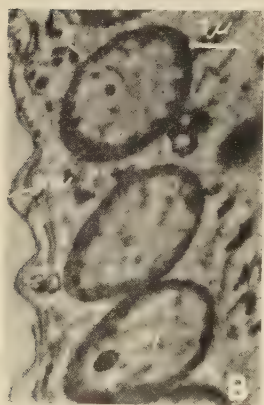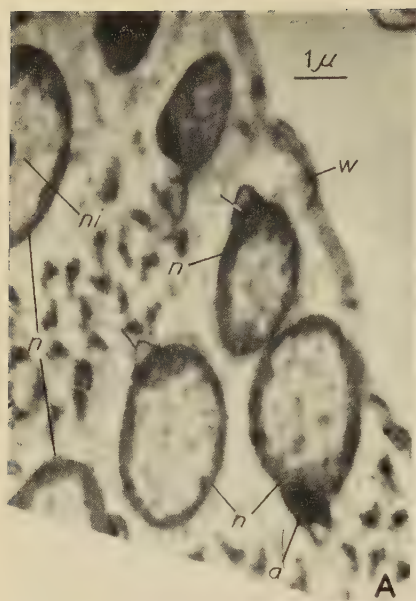
FIG. 5

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gave some results similar to, and others at variance to, those shown by the acroblast.

Under the electron microscope the acrosome appears at first to be almost structureless internally, though bounded by an electron-dense envelope (fig. 2, B). When it has reached the front end of the nucleus, its posterior end is very dense to electrons (fig. 5, A).

Nebenkern

The *Nebenkern* of the living cell, like the separate mitochondria of earlier stages, coloured strongly with dahlia and Janus green and black. Like the mitochondria it was coloured red by acid fuchsine in Metzner's technique.

It was darkened by osmium in the 'Golgi techniques', and with silver in the Aoyama technique. It reacted positively to Sudan black and feebly positive with acid haematein and PAS even after salivary extraction. The *Nebenkern* was feebly basiphil with basic fuchsine even after treatment with chromic acid.

The young *Nebenkern*, which, as we have seen, is formed by the aggregation of all the mitochondria in the cell, still retains something of the internal structure of the mitochondria (figs. 2, B; 4, A–C). Double membranes ('cristae') are seen within it, and some of them are clearly seen to extend to the wall of the organelle (fig. 4, A). Some of the electron micrographs show the *Nebenkern* in the act of dividing (figs. 4, B, C). The cristae are still present at this stage.

Fig. 5, C shows, in transverse section, the thin filaments into which the *Nebenkern* spins out at a later stage.

Axial filament

The electron micrographs show that the structure is the usual one for flagella and cilia: that is to say, there are two fibres near the centre and nine near the periphery (figs. 2, B; 5, C; 6, A). In one micrograph of the early stage (fig. 2, B) the eleven threads are clearly surrounded by a membrane. There is some indication that the fibres may be hollow.

Nucleus

The nucleus of the young spermatid (figs. 2, A, B; 3; 4, A–C) shows the granular structure usual in electron micrographs. The granules appear to be uniformly distributed. Some of the sections of the nuclei show a rounded electron-dense object, which may be the X-chromosome or possibly the

FIG. 5 (plate). A, spermatid, later stage. Wall of cyst (*w*); acrosome (*a*) with electron-dense posterior end; nuclei (*n*) with electron-dense object (*ni*).

B, spermatid, later stage. Wall of cyst (*w*); nuclei (*n*) with electron-dense object (*ni*).

C, axial filament (*f*); transverse section (left side) and longitudinal section (right side). Two filaments (*nk*) into which the *Nebenkern* spins out; cytoplasmic covering (*cc*); wall of cyst (*w*).

D, spermatid, late stage. Longitudinal section of nucleus showing radial structure and attachment of tail (*t*); dorsal (*d*); ventral (*v*).

nucleolus. The nucleus is surrounded by a double membrane (figs. 2, A; 4, B).

When the nucleus begins to elongate, the outer part of it becomes electron-dense (figs. 5, A, B). When the nucleus is greatly elongated in the late spermatid, an unusual radial structure appears in its outer part. This radial structure is well seen in a transverse section through the nucleus (fig. 6, A). At a still later stage the radial structure still appears in longitudinal sections of the nucleus (fig. 5, B). It is difficult to reconcile the appearance given in transverse and longitudinal sections. Longitudinal sections suggest the presence of transverse partitions extending into the interior from the nuclear membrane. The radial structure is not always seen in the nucleus of the late spermatid (fig. 6, B).

The attachment of the tail to the nucleus is shown in fig. 5, D. The nucleus is indented at the attachment and overhangs the base of the tail much further on one side than on the other.

The nucleus is enclosed by a thin cytoplasmic covering (fig. 6, B).

Discussion

In an electron microscope study of the male germ-cells of the cricket, *Nemobius*, Beams and others (1956) found that the acroblast consisted of double-membraned lamellae and constantly associated vesicles (externum) and an internal vacuolated ground substance (internum).

Although our study is concerned with the acroblast of a different species (*Acheta*), a strong correspondence exists between the structure of this cellular inclusion and that of the species mentioned above. Both exhibit a somewhat comparable shape in the early spermatid stage; both consist of parallel double-membraned lamellae and an internal finely vesicular region.

However, the number of lamellae in *Nemobius* is higher than in *Acheta* and their arrangement is more regular. In the former species, the external vacuoles are larger than those shown by *Acheta*, in which species they may be lacking.

Beams and others suggest that the lamellar region in *Nemobius* is probably comparable to the osmiophil region and the inner vacuolar area to the osmiophobe region of classical description. We have shown in *Acheta* that this suggestion has largely been verified by a combination of electron microscopy, histochemistry, and the so-called classical 'Golgi techniques' all carried out on the same tissue.

Fig. 6 (plate). A, spermatid, late stage. Transverse section of nucleus showing curious structure. Axial filament (*f*); filaments (*nk*) into which *Nebenkern* spins out.

B, spermatid, late stage. Cytoplasmic covering (*c*).
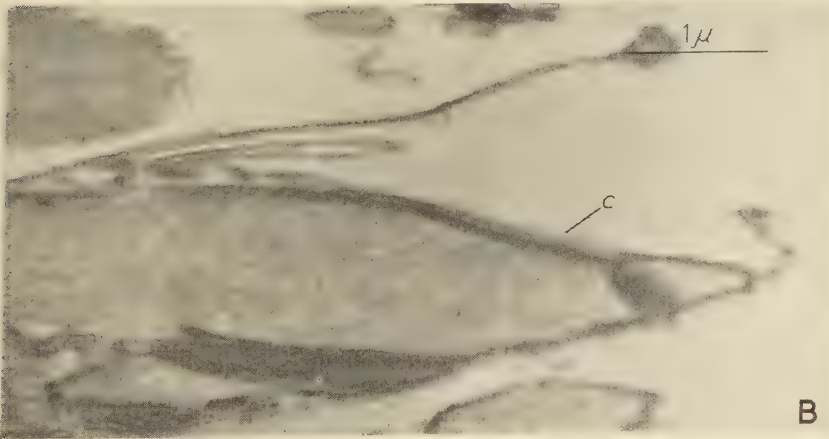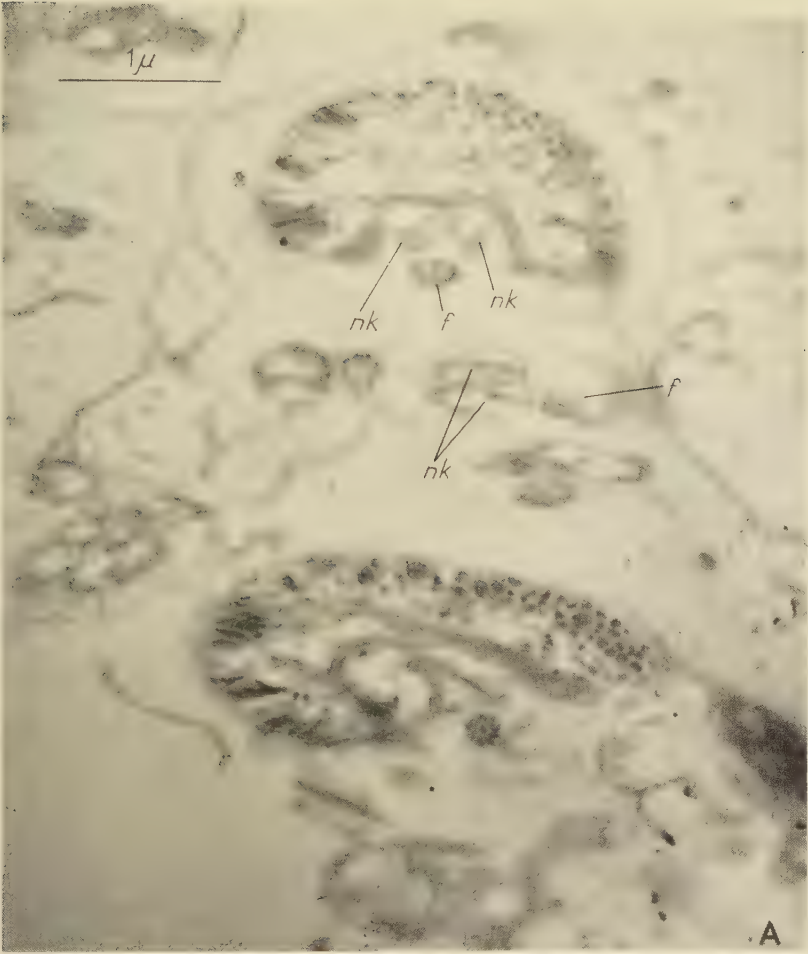
Fig. 6

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REFERENCES

AOYAMA, F., 1929. Z. wiss. Mikr., **46**, 489.
BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947. Ibid., **88**, 115.
—— 1949. Ibid., **90**, 293.
—— 1956. Ibid., **97**, 621.
—— 1957. Ibid. (in press).
BAUER, H., 1933. Jahrb. Morph. mikr. Anat., 2 Abt., **33**, 143.
BEAMS, H. W., TAHMISIAN, T. N., DEVINE, R. L., and ANDERSON, E., 1956. J. biophys. biochem. Cytol., **2**, no. 4, suppl., 123.
CAIN, A. J., 1947. Quart. J. micr. Sci., **88**, 383.
FEULGEN, R., and ROSSENBECK, H., 1924. Z. phys. Chem., **135**, 203.
HERXHEIMER, G. W., 1901. Deut. med. Woch., **36**, 607.
JORDAN, B. M., and BAKER, J. R., 1955. Quart. J. micr. Sci., **96**, 177.
—— 1956. Entomol., **89**, 126.
KOLATCHEV, A., 1916. Arch. russes d'anat. d'hist. et d'emb., **1**, 383.
LISON, L., 1953. *Histochimie et cytochimie animales*. Paris (Gauthier-Villars).
METZNER, R., and KRAUSE, R., 1928. In Abderhalden's *Handbuch der biologischen Arbeitsmethoden*, Abt. v, **2**: 1, 325.
NATH, V., and SINGH BHIMBER, B., 1953. Res. Bull. East Punjab Univ., **37**, 145.
PALADE, G. E., 1956. J. biophys. biochem. Cytol., **2**, no. 4, suppl., 85.
PEARSE, A. G. E., 1954. *Histochemistry, theoretical and applied*. London (Churchill).
WEIGL, R., 1910. Bull. internat. Acad. Sci. Cracovie, Sér. B. (no vol. no.), 691.

APPENDIX

A summary of the histochemistry of the spermatid of the house-cricket, Acheta domesticus

Test or technique	Practical notes				Results			
	Fixation	Embedding medium	Thickness of section in μ	Reference	Acroblast		Acrosome	Nebenkern
					Internum	Externum		
Living cells								
Methylene blue, 0·04%	—	—	—	—	0	0	0	0
Dahlia violet, 0·02%	—	—	—	—	0	+	0	+++
Neutral red, 0·02%	—	—	—	—	0	++	++	0
Janus black, 0·02%	—	—	—	—	0	+	+	+++
Janus green, 0·02%	—	—	—	—	0	0	0	+++
Thionine, 0·02%	—	—	—	—	0	0	0	0
Brilliant cresyl blue, 0·01%	—	—	—	—	0	0	0	0
Fixed material								
Standard Sudan black	FS+PC	G	10	Baker, 1944, 1949	0	++	0	++
Sudan black	L	G	10	Baker, 1956	+	++	0	+++
Sudan black	FCa+PC	G	10	Ibid.	0	++	0	+++
Sudan black	L+PC	G	10	Chou (unpublished)	+	++	0	++
Acid haematein	FCa+PC	G	10	Baker, 1946	0	++	+++	+
Acid haematein	L+PC	G	10	Chou (unpublished)	0	++	+++	++
Sudan IV	FS	G	15	Herxheimer, 1901	0	0	0	0
Nile blue	FS	G	15	Cain, 1947	0	0	0	0
Liebermann	FS	G	15	Lison, 1953	0	0	0	0
PAS	B, Z	P	10	Pearse, 1954	+	++	+++	+
PAS after saliva	B, Z	P	10	Ibid.	+	++	+++	+
Bauer	R	P	12	Bauer, 1933	0	0	0	0
Toluidine blue for meta-chromasy	B, Z	P	10	Baker (unpublished)	0	0	0	0
Toluidine blue after chromic acid, ¼, ½, 1 h	B, Z	P	10	Lison, 1953	++	++	+++	0
Toluidine blue after sulphuric acid	B / Z	P / P	10 / 10	Ibid.	++ / 0	+++ / 0	+++ / 0	0 / 0

Method	Fixative	Embedding	n	Reference					
Basic fuchsine for basiphilia	B	P	10	—	O	O	O	O	++
	Z	P	10	—	O	O	O	O	O
Basic fuchsine after chromic acid, ¼ h	B	P	10	—	O	O	O	O	++
Pyronin and methyl green	Z, 3 h	P	7	Jordan and Baker, 1955	O	O	O	O	O
Feulgen	Z	P	10	Feulgen and Rossenbeck, 1924	O	O	O	O	O
Sakaguchi	Z	P	10	Baker, 1947	+O	+O	+O	+O	+O
Windaus	FS	G	15	Lison, 1953	O	O	O	O	O
Weigl (Mann–Kopsch)	Mann, PO 8 days	P	4	Weigl, 1910	++	+++	++	O	++
Kolatchev	Champy PO	P	4	Kolatchev, 1916	+O	+++	++	O	++
Kolatchev	Meves PO	P	4	Ibid.	++	++++	++	O	++.+
Aoyama (toned and untoned)	Aoyama	P	4	Aoyama, 1929	+O	++++	++	O	++
Baker (Hermann–Kopsch)	Hermann PO	P	4	Baker, 1957	O	+++	O	O	+++
Metzner	A	P	3	Metzner and Krause, 1928	++	O	++	++	++++
Metzner	H+PC	P?	3	Ibid.	O	O	+++	+++	++

KEY: A = Altmann's fluid; B = Bouin's fluid; FCa = formaldehyde calcium; FS = formaldehyde-saline; G = gelatine; H = Helly's fluid; L = Lewitsky-saline; P = paraffin; PC = postchromed; PO = postosmicated; R = Rossmann's fluid; Z = Zenker's fluid; 3 h = 3 hours; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative.

The Absorption of Oleic Acid from the Small Intestine of the Rat

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With one plate (fig. 1)

SUMMARY

By the use of histochemical techniques, the lipids (fatty acid, neutral fat, and phospholipid) were studied in the villi of the small intestine of the rat, 1 h and $2\frac{3}{4}$ h after feeding with oleic acid. The results of these experiments were identical with those previously described when triolein was administered. Neutral fat, fatty acid, and phospholipid were present in varying quantities within the cells. Between the cells and in their striated border, neutral fat and fatty acid were detectable. Within the cells, fatty acid was most abundant 1 h after feeding, whereas neutral fat and phospholipid predominated $2\frac{3}{4}$ h later. The direct relationship between the amounts of neutral fat and phospholipid present in the cells, which had been observed in the studies with triolein, prevailed in the experiments described here.

INTRODUCTION

DURING the absorption of neutral fat, phospholipid appears in the epithelial cells of the small intestine. It has been suggested that this substance is concerned with the reformation of the fat, which had undergone hydrolysis in the intestinal lumen. In previous experiments (Hewitt, 1956) neutral fat, fatty acid, and phospholipid were detected in varying proportions in the intestinal epithelial cells of rats which had previously been fed with triolein. There appeared to be a direct relationship between the amounts of neutral fat and phospholipid present and it was suggested this might indicate a link between them. It was possible, however, that much of the neutral fat had not been synthesized but was actually unhydrolysed fat which had been absorbed as such. To exclude this the experiments were repeated, but each animal was fed on 0·18 ml of oleic acid instead of triolein. The average weight of the rats used was 200 g. A group of animals was killed, as before, $2\frac{3}{4}$ h after feeding and an additional group was killed after 1 h. Apart from these differences the materials and histochemical techniques employed for detecting lipid, neutral fat, fatty acid, and phospholipid were the same as those previously described and reference should be made to this description for details.

RESULTS

The results obtained were indistinguishable from those obtained after feeding with triolein. Neutral fat and fatty acid were detected between the epithelial cells, in their striated border, and within them, where phospholipid was also present. As before, the intracellular content of these three substances

was variable, but there were marked differences in their relative proportions in the two groups of experiments. In the group killed 1 h after feeding, fatty acid predominated in the cells with only small amounts of neutral fat and phospholipid present. In the other group the reverse prevailed: there was a preponderance of neutral fat and phospholipid with the complete absence or only minimal amounts of fatty acid. The same relationship was also observed between the amounts of neutral fat and phospholipid present, as in the previous experiments, and this was even better demonstrated by comparing the two groups of results (fig. 1).

DISCUSSION

It was tempting to conclude from these observations that phospholipid formation was essential for the formation of the neutral fat which appeared within the epithelial cells. However, no support for this was provided by these results. The formation of phospholipid and neutral fat may have been occurring at the same time and merely delayed until the mobilization of their other components, the glycerol fraction of which was common to them both. It was not unreasonable to assume that the neutral fat had been formed from the ingested fatty acid and that this occurred within the epithelial cells. The synthesis could, however, have been in the intestinal lumen before absorption and might have explained the presence of neutral fat both between the cells and in their striated border. The possibility, however unlikely, that some or even all of the neutral fat was not synthetic but had been derived from other sources should not be overlooked. It could have been derived from the intestinal lumen or from the lymphatic or blood vessels of the villi. The presumption is made that the flow of lipid is from the intestinal lumen towards the core of the villus; but in static preparations there is nothing to indicate the direction of movement of the particles, and the possibility of a two-way flow should be borne in mind. It may well be that some of the lipid seen was travelling towards the intestinal lumen into, through, or between the epithelial

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FIG. 1 (plate). Photomicrographs of 10-μ sections of villi from the small intestine of the rat.

A, Sudan black preparation, showing the location of the lipid in two villi 1 h after feeding with oleic acid. The majority of the lipid within the cells consists of fatty acid with only small quantities of neutral fat and phospholipid.

B, Sudan black preparation, showing the location of the lipid in two villi 2¾ h after feeding with oleic acid. Apart from the lipid seen in the core of the villi and in the cells deep to their nuclei, the appearances are similar to A. This lipid, however, consists of neutral fat and phospholipid.

C, Fischler preparation. Section of a villus from the same segment of intestine as A, showing that much of the lipid consists of fatty acid.

D, Fischler preparation. Section of a villus from the same segment of intestine as B, showing the absence of fatty acid.

E, acid haematein preparation. Section of villi from the same segment of intestine as A and C. Only a small amount of phospholipid can be seen in the cells around the site of the Golgi apparatus.

F, acid haematein preparation. Section of villus from the same segment of intestine as B and D. In contrast with E, the phospholipid content of the epithelial cells is much higher, particularly deep to their nuclei.

50μ

FIG. 1

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cells. It is conceivable, therefore, that some of the neutral fat was mono- or di-glyceride from the animal's own resources, which by combining with the incoming oleic acid formed a mixed triglyceride. This would avoid the necessity of providing glycerol for the synthesis of triolein; a problem discussed by Frazer, Schneider, and Sammons (1956), who gave references.

Although these results are of interest, they neither confirm nor disprove the theory that phospholipid formation is an essential part of triglyceride fat absorption. However, they provide a basis for further investigations which are being undertaken.

REFERENCES

FRAZER, A. C., SCHNEIDER, R., and SAMMONS, H. G., 1956. Gastroenterologia, **85**, 146.
HEWITT, W., 1956. Quart. J. micr. Sci., **97**, 199.

The Formation of Tracheae and Tracheoles in *Rhodnius prolixus*

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With one plate (fig. 1)

SUMMARY

The formation of tracheae in *Rhodnius* is described by the hypothesis of expansion and buckling. The cuticulin lining is at first a smooth-walled cylinder. Later it expands equally in each direction, increasing in diameter but buckling in the axis. An engineering expression describing symmetrical buckling in a cylinder under uniform axial compression has been applied to this process. Agreement was obtained between the expected and observed values for buckling frequency and tube-wall thickness. The taenidia are formed within the buckles, their amplitude being proportional to the increase in diameter. The axial orientation of the chitin micelles in the lining membrane and the tangential orientation in the taenidia are consistent with their being oriented by the stresses expected during expansion and buckling. The formation of tracheoles may also be described by the expansion and buckling hypothesis.

INTRODUCTION

THE formation of tracheae has been discussed by Wigglesworth, (1931), Richards and Anderson (1942), Keister (1948), Richards and Korda (1950), and Richards (1951).

The characteristic pattern in the cuticle of tracheae and the origin of the taenidia have attracted the attention of numerous writers from the earliest microscopists onwards. Thompson (1929) was the first worker whose approach agreed with modern ideas. He said: 'Since the tracheal filament is continuous, the natural supposition is that it results from the operation of some simple physical laws and is produced by forces which are unaffected by the existence of cell boundaries in the tracheal epithelium, and act simply in the chitinous lining at the moment when it is being secreted.' Keister (1948) studied the development of tracheae in living *Sciara* larvae with a phase-contrast microscope but did not arrive at any hypothesis to account for their formation. The events she described are exactly those expected from the hypothesis of tracheal formation put forward in this paper. Wigglesworth (1954) has described moulting in *Rhodnius* tracheae. He compared the folding of the lining with the expansion of the epicuticle over the rest of the body, saying that the taenidia are a secondary secretion within the folds. Richards had previously rejected this hypothesis because of his belief in the presence of a continuous basal procuticle. No such layer is present in *Rhodnius* and the hypothesis of expansion is here elaborated. In *Rhodnius* the tracheae consist of a two-layered lining membrane folded round three sides of the taenidia, the annular or

helical thickenings which separate the membrane from the epithelium (fig. 1, C). Both the outer layer of the membrane and the taenidia contain chitin, in the former with the micelles axially oriented and in the latter with the micelles lying tangentially (Locke, 1957). The problem is to account for (1) the regular helical and annular folding of the cuticle, (2) the formation of the taenidia and their micelle orientation, and (3) the axial orientation of micelles in the lining membrane.

The advent of the electron microscope has shown that tracheoles have a structure similar to that of tracheae—a lining membrane supported by annular or helical thickenings, but too small for the micelle orientations to be determined. This obvious similarity between tracheae and tracheoles suggested that the same mechanism might be operating in their growth.

MATERIAL AND METHODS

At 25° C 4th instar larvae of *Rhodnius prolixus* Ståhl moult 14–15 days after feeding. A series showing stages in the formation of a trachea was obtained by fixing at intervals. The usual procedure was to dissect under Ringer and to fix and stain before removing the insect from the dissecting dish for the preparation of a whole mount. In this way measurements were taken from tracheae left in their natural position with a minimum of distortion. Aqueous Bouin gave satisfactory results. Electron micrographs were taken at the Cavendish laboratory with a Siemens Elmiskop I electron microscope. For this purpose tracheae were fixed in 1% osmium tetroxide buffered at pH 7·4 and embedded in 1:1 butyl / methyl methacrylate.

RESULTS

The epithelium and ecdysial membrane

The cells begin to divide on the 3rd to 4th day after feeding and continue to multiply until the 7th day. Nuclear destruction begins on the 4th to 5th day, reaches a peak by the 8th day, and is almost complete by the 9th day, when the new cuticle is apparent with the light microscope. The cytoplasm increases slightly in thickness with the increasing density of nuclei until the 7th day when the change is more abrupt. Fig. 1, A, B show the great activity of the cells at this time with abundant inclusions.

The slight withdrawal of the thickened epithelium which occurs from the 7th day is associated with the formation of what will be described as an ecdysial membrane, although some of its properties differ from the membrane described by Passoneau and Williams (1953) and Richards (1955). On the 7th day the inner cell-membrane appears thickened. By the 8th day the membrane appears in optical section as a thin PAS-positive line between the epithelium and the old trachea. At this stage it is not uniform: in surface view it is seen to be longitudinally striated (fig. 1, J). This is not an artifact of fixation, for the same striation is seen under phase contrast in fresh material. The reticulate nature of the membrane is well shown in sections under the

FIG. 1

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electron microscope (fig. 1, A). This striation persists until the new trachea has expanded some way from the old. It is most readily seen just as the new cuticle is buckling but it is certainly present before then. By the time the new trachea has reached its final diameter the ecdysial membrane is much more uniform (fig. 1, I). A sheet of it under the electron microscope appears without striations.

The membrane differs from ecdysial membranes described previously in that it has not been detected in the exuviae. It dissolves in the moulting fluid before ecdysis. Nor is it as resistant to strong alkali or diaphanol. Even after fixation it dissolves or is lost. It must be a different sort of carbohydrate from the chitin-protein complex of the outer layer of the lining membrane.

The pattern formed by the expansion of the lining membrane

When the ecdysial membrane has separated, the epithelium secretes the first layer of the new cuticle. By 8 to 9 days this layer shows up sharply in electron micrographs; it increases rapidly in area, buckling in the axis while the epithelium separates from the old trachea to take up the new diameter. Some phenomena may be interpreted as indicating that the epithelium is subjected to pressure from within at this stage. The equilibrium between the epithelium and the new cuticle appears to be upset in some preparations after fixation. The newly buckled cuticle takes up a position as if it were a broad helical spring forced to contract axially with asymmetry by a narrow central cord (fig. 2). It looks as if the expanding cuticle is forcing the epithelium to

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Fig. 1 (plate). All are electron micrographs except I and J, and all except D and E are of *Rhodnius*. All the electron micrographs are longitudinal sections except F and M, which are whole mounts.

A, 4th instar trachea 8 days after feeding. The ecdysial membrane appears as an interrupted line. The new cuticle is only just visible.

B, 9 days after feeding, showing the 5th instar trachea only. The new cuticle has just begun to buckle. The amplitude of the waves is slightly exaggerated by the oblique plane of the section.

C, single taenidium from a mature 5th instar trachea.

D, part of a taenidium and the epicuticle from a cockroach trachea.

E, the same for a mealworm larva. The epicuticle lining the inner face of the taenidium is similar to that between the taenidia but without the tubercles.

F, carbon replica of a tracheole from a 5th instar larva. The taenidia stand out as raised ridges.

G, a tracheole during its formation in the testis membrane of a 4th instar larva, 10 days after feeding. The tracheoblast cell-membrane lies along the bottom of the picture.

H, a mature tracheole from the same situation.

I, optical section with a light microscope of a trachea from a 4th instar larva, 12 days after feeding, showing the old and new tracheae and between them the ecdysial membrane.

J, Surface view of a 4th instar trachea, 8 days after feeding, focused between the nuclei and the old trachea. The ecdysial membrane appears as a series of striations diagonally from the bottom left of the picture at right angles to the taenidia.

K, 5th instar trachea, showing the formation of a taenidium. The cell-membrane has withdrawn from the inflated fold and material is gathering in the flattening tip.

L, a small part of G greatly enlarged to show the sheath-membrane round the buckling lining.

M, tracheoles showing the normal taenidial frequency.

take up the increased diameter of the new trachea. This is the increase in area
of the cuticle to which Wigglesworth (1954) refers. The expansion is equal
in the axis and the circumference. The outline of the cuticle seen in optical
section was drawn with a camera lucida in a number of preparations of large
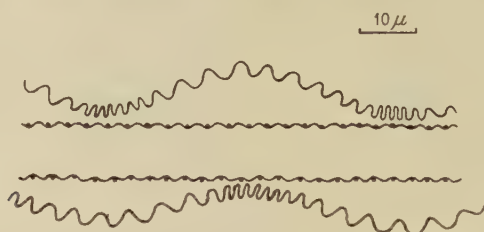
FIG. 2. The outline of the old 4th and new 5th instar tracheal
cuticle in a larva 10 days after feeding, when the equilibrium
between the new cuticle and the epithelium has been upset by
poor fixation.

tracheae which show various stages of buckling. The axial expansion was
then measured on the drawing with a cyclometer. Fig. 3 shows these data
plotted against the increase in diameter. There is very good agreement with
the hypothesis that the cuticle is expanding uniformly in the axis and the
circumference.

Many authors have referred to the folding of the new cuticle (Wigglesworth,
1933; Wolfe, 1954), but the concept of buckling in response to stress has not
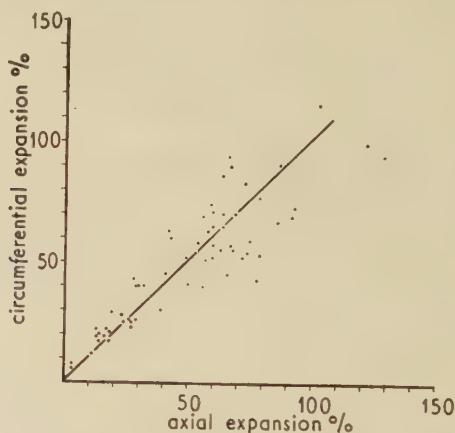
FIG. 3. The uniform expansion of the new tracheal cuticle in
the length and circumference of the tube.

previously been introduced. The uniform expansion of the tracheal cuticle
and the simple regular nature of the resulting deformation suggest that the
cuticle is isotropic at this stage. The cuticle is also approximately constant in
thickness in tracheae of different size and it is probably elastic (the completed
two-layered membrane in the exuviae tends to lose the taenidial folds and the

isolated cuticulin layer alone shows scarcely any deformation). It is therefore reasonable to suppose that the newly formed cuticular tubes of the tracheae can be treated as thin-walled elastic cylinders with uniform properties varying only in radius. The tracheal cells also form a layer approximately uniform in thickness without any obvious anisotropy. Little is known about the elasticity

wall stress (S) × wall area = fluid pressure (p) × fluid section area

$$S(2\Delta lt) = p(2\Delta lr)$$
$$S = \frac{pr}{t}$$

$$S(2\pi rt) = p(\pi r^2)$$
$$S = \frac{pr}{2t}$$
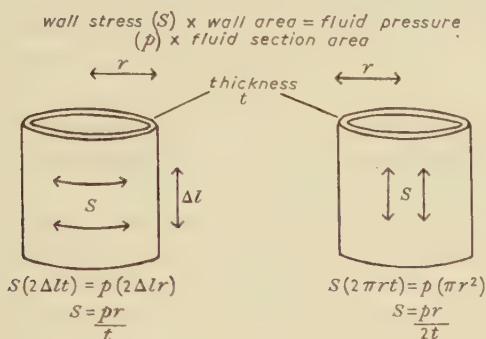
FIG. 4. Diagram showing how in a cylinder under uniform internal pressure the hoop stress is twice the axial stress. (After Castle, 1937.)

of such cells but it is probably low (Mitchison and Swann, 1954). The problem is to account for the buckling produced in the inner elastic cylinder when it expands against the outer one.

Now in a cylinder under uniform internal pressure the hoop stress is twice the axial stress (Timoshenko, 1936, p. 479). This was brought to the notice of biologists by Castle (1937). A cylindrical boiler blows up by tearing along the axis rather than by blowing off its ends (Fig. 4). The cuticle expands uniformly. Thus the restraining epithelium will tend to give first in the circumference. It need not give at all axially unless the critical axial stress for deformation in the cuticle exceeds twice the critical circumferential stress in the epithelium. Thus by balancing the critical strength of the cuticle between the critical stresses for axial and circumferential deformation of the epithelium, axial buckling alone could be produced. It may not be legitimate to refer to critical stresses in an epithelial layer in this way. The source of restraint is unknown. It is not likely that the old trachea embedded in the gel of moulting fluid contributes to the axial restraint since tracheae not forming round existing tracheae have a normal structure. The restraint may reside partly in the inner cell-membrane although this is unlikely since the membrane at first follows the buckles (fig. 1, B). For convenience the whole epithelium has been referred to as the source of the restraint since it appears to be the expanding cuticle which causes it to increase in diameter. The ultrathin cuticle might seem too fragile to move an epithelial layer 100 times as thick, but once axial buckling has occurred its resistance to lateral deformation increases. It may then be compared to a cylinder with stiffened rings. A knowledge of the Young's modulus of the epithelium would not be useful without information about its variation with time and stress. A layer of this sort might be markedly elastic

to sudden deformation, but over the hours or days of tracheal formation its behaviour might be almost plastic.

From Mitchison and Swann's work (1954) it is unlikely that cell-membranes respond linearly to stress. If these membranes and the whole epithelium did respond linearly over a wide range, then some axial extension of the cuticle should be observed before buckling. This is not detectable in practice. Buckling takes place almost as soon as the cuticle can be observed to be separate from the ecdysial membrane. This also indicates that the cuticle is appreciably elastic.

If the epithelial restraint had no yield point below the critical stress for circumferential deformation of the cuticle, then the system would be comparable to a cuticular tube under uniform external pressure (see Timoshenko, 1936, p. 479). Under these conditions the tube deforms asymmetrically, the number of lateral lobes depending on the ratios of length and wall thickness to diameter. Tracheae have not been reported with this deformation, but a slightly less regular buckling of this type is found on the nodes (junctions between tracheae from different regions). This pattern may be simulated by lining a glass capillary tube with rubber latex and causing it to swell in xylene. Edwards and others (1954) note but do not figure a tracheole with crossed helical thickenings. This also might well result from a restraint too great for the cuticle.

Whatever the critical stress and the precise origin of the restraint, the result in normal tracheae is buckling symmetrical with respect to the axis (helices are discussed latter). This would be expected from the foregoing discussion.

The frequency of buckling

Buckling in a cylinder which is expanding under axial restraint should be comparable to buckling in a cylinder under uniform axial compression (fig. 5). Symmetrical buckling would be expected in biological material with a low Young's modulus. In symmetrical buckling the buckling frequency, n, is related to the initial radius r, the wall thickness t, and Poisson's ratio (v) for the material, in the expression

$$1/n = \frac{\pi\sqrt{(rt)}}{\sqrt[4]{\{12(1-v^2)\}}} \quad \text{(Timoshenko, 1936, p. 441).} \quad (1)$$

Poisson's ratio (v) must lie between $+\frac{1}{2}$ and -1. v for steel $= 0\cdot3$, and for rubber $v = 0\cdot46-0\cdot49$ (Kaye and Laby, 1948), so for biological materials v should be close to $0\cdot5$.

For $v = 0\cdot3$, $\qquad\qquad\qquad \frac{\pi}{\sqrt[4]{\{12(1-v^2)\}}} = 1\cdot72,$

and $v = 0\cdot5$, $\qquad\qquad\quad \frac{\pi}{\sqrt[4]{\{12(1-v^2)\}}} = 1\cdot81.$

Thus there should be very little error in simplifying equation (1) above to

$$1/n = 1 \cdot 8 \sqrt{(rt)}. \qquad (2)$$

Now if the formation of taenidial folds is comparable to buckling, it should be possible to describe it by this formula. *n*, the number of half-waves of buckling per unit length, is known and by using preparations similar to fig. 1, 1, the initial radius (*r*) may also be measured. If log *r* is plotted against log *n* for tracheae of different size, the result should be a straight line of slope $-\frac{1}{2}$, the position of the line being fixed by the value of *t*, the initial thickness. Fig. 6, A shows such a graph for *Rhodnius* 5th instar tracheae taken just before emergence from the 4th instar exuvium. The slope is a fair approximation to $-\frac{1}{2}$. Fig. 6, B, C, D gives similar graphs for tracheae from intermoult 3rd and 4th instar *Rhodnius* and from the nymphal cockroach, *Periplaneta*. *r* here is the final radius of the trachea, but since the increase in diameter is not a function of the radius this should not have altered the slope. These graphs also show a good approximation to the slope predicted by theory.

FIG. 5. The formation of buckles in an expanding cylinder restrained in the axis (A) should be comparable to buckling in a cylinder under uniform axial compression (B).

In fig. 6, A, C, D the slope is slightly less than $-\frac{1}{2}$. This error would occur if the taenidia were farther apart than theory predicted by an amount inversely proportional to *r*. Now the buckling frequency has been measured upon tracheae with completely formed taenidia. It was assumed with Keister (1948) that the spacing of taenidia did not change during their formation, but this is probably not quite correct. In some tracheae the amplitude of the waves is so large that the original buckles must have been forced apart as the cuticle continued to expand. In tracheae from the testis which increase in diameter by up to 300% this has certainly happened. This axial expansion after buckling will tend to be inversely proportional to the radius, since a small trachea will have relatively thicker walls and will be proportionally resistant to compression.

If this interpretation is correct, data taken from newly buckled tracheae, or from tracheae which have increased little in diameter, should show an even closer approximation to the theoretical slope of $-\frac{1}{2}$ than fig. 6, A–D. Accordingly the buckling frequency was measured on tracheae before taenidial formation was complete. Fig 6 E shows that the buckling frequency is then very close to the theoretical indeed. The amplitude of buckling waves is also very small in intermoult *Tenebrio* larvae. Fig. 6, F shows that here also the slope is close to that predicted by theory.

Thus as far as the slope of the graphs is concerned there is a very plausible agreement with the theory that taenidia result from buckling of the cuticle. The data are summarized in table 1.
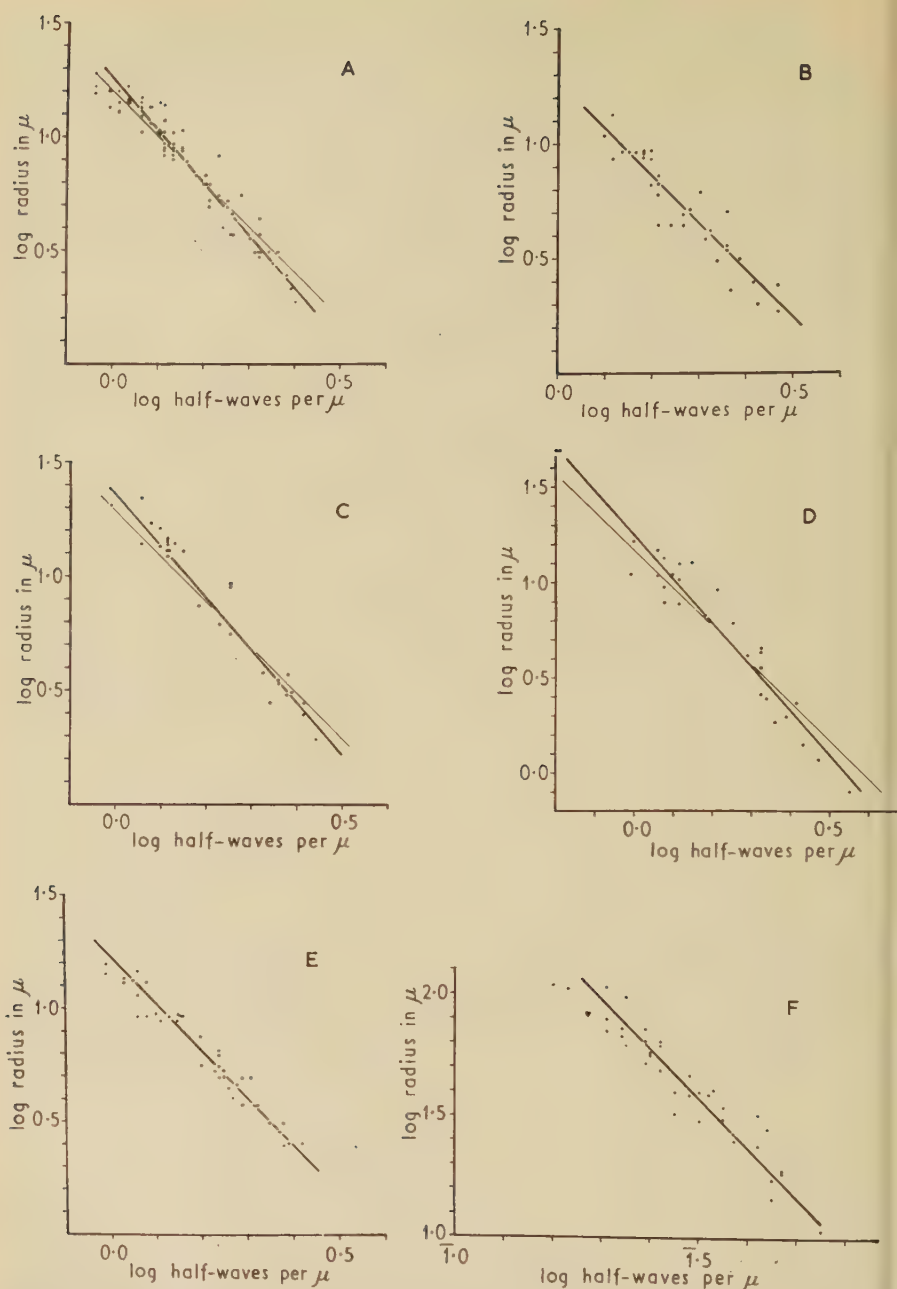
Fig. 6. The relation between taenidial frequency and the radius of tracheae. A, 4th instar
Rhodnius larvae just before moulting to the 5th instar. B, 3rd instar *Rhodnius*. C, 4th instar
Rhodnius. D, nymphal cockroach. E, 4th instar *Rhodnius* larvae, 10 days after feeding, before
expansion of the new 5th instar trachea is complete. F, *Tenebrio* larvae. Ordinate: B, C, D, F,
log radius of the trachea; A, E, log radius of the old trachea. Abscissa: B, C, D, F, log number of
taenidia per 2 μ; A, log number of taenidia per 2 μ in the new 5th instar trachea; E, log half-
waves of buckling per 1 μ in the new 5th instar trachea. A slope of $-\frac{1}{2}$ is indicated by a thin
line where this deviates sufficiently from the best-fitting straight line for the data.

The thickness of the cuticle

Since the thickness t is proportional to $(1/n)^2$, quite small errors in the measurement of the spacing of the taenidia will have a marked effect upon estimates of the thickness. Such errors might tend to affect tracheae of all sizes equally so that the fit of the slope is not necessarily an indication of the accuracy of the estimate of thickness. The standard deviations in table 1 indicate the precision of the agreement with the slope of $-\frac{1}{2}$ rather than the accuracy of the thickness estimate.

TABLE 1

Figure	Data from	Correlation coefficient	Slope	Thickness and S.D.	Diameter increase	Probable diameter increase	Thickness and S.D.
				(Å)	(%)	(%)	(Å)
5, A	5th instar Rhodnius, taenidia before ecdysis	−0·95	−0·43	197 34	67	..	..
5, E	5th instar Rhodnius, newly buckled tracheae	−0·97	−0·48	196 27	48	..	..
5, F	Intermoult Tenebrio	−0·83	−0·48	..	..	20	1,000 205
5, B	Intermoult 3rd instar Rhodnius	−0·94	−0·49	..	..	50	266 50
5, D	Intermoult Periplaneta	−0·97	−0·43	..	..	50	311 90
5, C	Intermoult 4th instar Rhodnius	−0·97	−0·44	..	..	50	239 50

The initial thickness of the cuticle calculated from the data in fig. 6, E is 196 Å, S.D. 27 Å. This estimate has been verified approximately with the electron microscope. Preparations before buckling have not been used for this since they may not have reached their final thickness preparatory to expansion. Fig. 1, B shows a large trachea which has just begun to buckle. The amplitude of the buckles is exaggerated in this preparation because the section does not pass exactly down the meridian of the tube. Measurements of the thickness of the cuticle at this stage lie between 160 and 200 Å. The most precise measurements from enlarged plates taken at a magnification of 20,000 suggest a thickness of about 180 Å. This is very close to the predicted value. The cell-membrane follows the buckled cuticle and perhaps contributes to the calculated thickness. This would account for the slightly lower value of the

measurement, but it is not possible from the photographs available to say that the measured thickness differs significantly from that calculated. Most of the other errors tend to make the calculated thickness maximal. The wider spacing of taenidia than first-formed buckles has already been mentioned. The magnitude of the initial circumferential restraint is unknown but it would also tend to depress the buckling frequency.

Calculations of thickness from the data in fig. 6, B, C, D, F would tend to exaggerate these errors since tracheae elongate slightly upon release from the exuvium. These data are also less reliable, since a value for the initial radius has to be assumed for the calculation. A poor estimate for this can be made from the amplitude of the folds, but this is not reliable since it will be affected by the extension of the trachea at ecdysis. The figures in the last column in table I are therefore included only to show that they do not greatly conflict with the more carefully obtained data for 5th instar *Rhodnius*. All values are high, as expected. It is perhaps significant that electron micrographs of mature tracheae of *Tenebrio* and *Periplaneta* show a thicker cuticle than 4th or 5th instar *Rhodnius* (fig. 1, D, E).

The formation of helices

The previous argument has been simplified by treating the low-pitch helices which occur in large tracheae as if they were annuli. It seemed likely, as Richards (1951) suggested, that a tangential shearing force added to the forces responsible for annular taenidial formation might cause a helix to form. The critical normal stress A_{cr} necessary to produce annular buckling from axial compression is given by

$$A_{cr} = \frac{Et}{r\sqrt{\{3(1-v^2)\}}} \quad \text{(Timoshenko, 1936, p. 441),} \tag{3}$$

where E = Young's modulus and v = Poisson's ratio for the material of the tube-wall of thickness t and radius r. Putting in reasonable values for v ($= 0.48$), t ($= 0.02\,\mu$), and r ($= 20\,\mu$), this becomes

$$A_{cr} = \frac{0.02E}{20\sqrt{\{3(1-0.48^2)\}}} = \frac{0.02E}{30.4} = 7 \times 10^{-4}E.$$

The critical shear necessary to produce helical buckling is given by

$$T_{cr} = \frac{E}{3\sqrt{2}(1-v^2)^{\frac{3}{4}}}(t/r)^{\frac{3}{2}} \quad \text{(Timoshenko, 1936, p. 486).} \tag{4}$$

Putting in the same values as before this becomes

$$T_{cr} = \frac{E}{3\sqrt{2}(1-0.48^2)^{\frac{3}{4}}}(0.02/20)^{\frac{3}{2}} = \frac{E}{3.49} \times 3.2 \times 10^{-5} = 9 \times 10^{-6}E.$$

Thus the critical shearing stress necessary to produce helical buckling is only a very small fraction of the axial stress necessary to produce annular buckling. Helical folds in tracheae are not comparable to those produced by

torsion alone, for this induces helices with a high pitch with lobes in the circumference rather than the axis. The low-pitched spirals of tracheae probably result from an axial stress with only a slight shear component. This may be illustrated by a model. A thin rubber sleeve can be prepared by allowing rubber latex to dry over a glass rod. If this is lubricated with water, it will slide and fold freely on the rod. Axial compression on the rubber induces the formation of annuli, while twisting produces helices. Compression with a very slight twist produces helices with about the same pitch as are found in tracheae.

Thus the torsion necessary to change annuli into helices is very small. It seems probable that randomly-occurring torsional stresses in the tissues could be responsible. The distribution of helices in tubes of different diameter supports this. Whereas the critical axial stress is proportional to the ratio of wall thickness to radius, the critical shear stress is proportional to this ratio[2]. Thus while the critical shear may be only just over 1% of the critical axial stress in tubes of radius $20\,\mu$, it is over 8% in tubes of radius $1\,\mu$. Small tubes are almost always annular and large tubes almost always helical.

The control of expansion

It will be shown in a future paper that adjacent parts of a trachea may increase in diameter by different amounts. Thus the agent controlling expansion is unlikely to reside in the moulting fluid. Either the extent of expansion is intrinsic in the membrane when first formed and is released to completion (perhaps by the moulting 'fluid'—Passoneau and Williams (1953) found that the composition of the 'fluid' changes with time; initially it contains tyrosinase, for example), or the expansion is controlled through the epithelium. If the former hypothesis were correct, then it might be expected that the initial thickness of the membrane would vary with its future diameter. There is some evidence that this is so. If the initial thickness calculated from measurements of taenidial frequency and radius as in fig. 6, A and table 1 is plotted against the increase in diameter, there is a significant positive correlation. But this could be and probably is the result of the poor fit for slope caused by the slight axial expansion during taenidial formation. This criticism could be discounted to some extent if the thickness calculated from mature tracheae with a very small increase in diameter proved to be significantly less than that calculated (fig. 6, E) and approximately verified with the electron microscope for normal tracheae.

Fourth instar larvae were induced to moult without feeding by joining in artificial parabiosis with fed larvae (Wigglesworth, 1934). Preparations were made as in fig. 1, I. The taenidia are fully formed but the amplitude of the waves is commensurate with the small increase in diameter (mean $= 35\%$). The thickness calculated from these tracheae is only 162 Å, S.D. 47 Å, which is significantly lower than normal ($P =$ less than 0.1%). Full weight should not be given to this result because of the small change in taenidial frequency which it represents, but it is suggestive. Now these preparations are highly abnormal. An insect forced to moult without adequate reserves might very

well lay down thinner membranes whether or not the future diameter depended on it. It is unwise to infer that the initial membrane thickness is proportional to the future diameter in the normal variations in growth. Nevertheless, the result does not conflict with the hypothesis that the future diameter is latent in the first formed membrane.

The formation of the taenidia

The spacing of taenidia in tracheae of different size depends upon the buckling of the cuticle already considered. The size of the taenidia depends
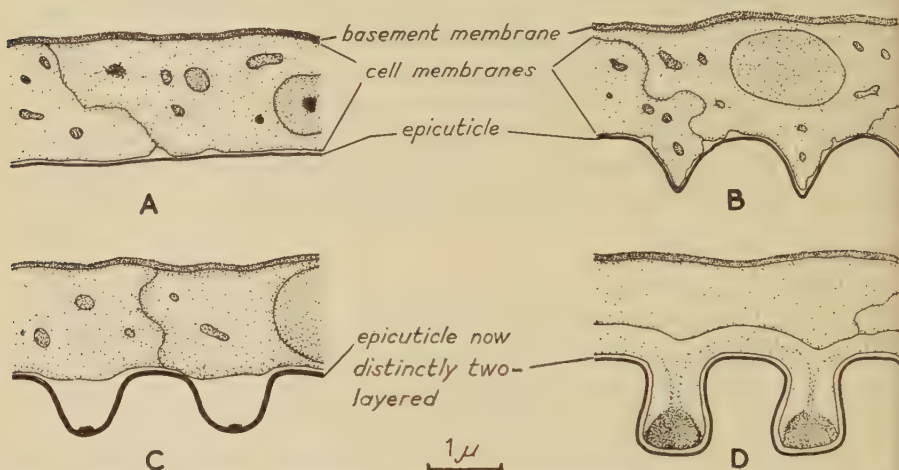
FIG. 7. Diagram showing the formation of the taenidia as seen in longitudinal section. A, the cuticulin is smooth as in fig. 1, A. B, it has begun to buckle as in fig. 1, B. C, the folds have inflated and the apices of the buckles have flattened with the deposition of the new taenidia as in fig. 1, K. D, the appearance of mature taenidia with flattened inner faces as in fig. 1, C.

upon the expansion. A series of large tracheae showing a range of increases in diameter was prepared by feeding the larvae to a varying extent. From these it was clear that the amplitude of the waves in mature tracheae varied with the diameter increase.

Fig. 1, A, B, K, C shows the progress of buckling. When the trachea has almost reached its final diameter the folds inflate with the deposition of the taenidia. In the first-formed buckles the cell-membranes follow the line of the expanding cuticle (fig. 7, B), but as expansion proceeds the cell-membranes are withdrawn, leaving clear spaces in which the taenidia are deposited (fig. 7, C). Now the expansion of the circumference will tend to exert a torque upon elongated particles free to move in these spaces so that they tend to build up in an oriented band at the apex of the folds. The electron micrographs give the impression that this is happening. With further expansion more material is laid down or crystallizes out, tailing off towards the epithelium (fig. 7, D). The formation of tangentially oriented bands would be expected to exert a considerable restraint upon the cuticle expanding in the circumference. In agreement with this the tubercles which probably have

their origin in the tiny irregularities at first seen all over the cuticle, are 'ironed out' over the inner surface of the taenidia where it tends to be flattened rather than rounded.

The appearance of the taenidia and the orientation of their micelles would be expected from the secretion of an endocuticle over an expanding buckled surface.

The orientation of chitin micelles in the lining membrane

Long molecules in thin films become oriented in fibrils at right angles to a lateral pressure (Tachibana and others, 1955). Thus the axial orientation of chitin micelles in the tracheal cuticle could be the result of lateral pressure. The time when this occurs is not known, since neither the taenidia nor the lining membrane withstand treatment for chitin purification very well during the early stages of their formation. The new cuticle when first formed appears as a thin sharply defined electron-opaque line. This has not been resolved into a double layer as in a cell-membrane; if anything it appears granular. During expansion it becomes thicker and by the time the epithelium has withdrawn from the folds it is distinctly two-layered as in mature tracheae. The first-formed cuticle seems to be continuous in time with the lining cuticulin layer, the main change during expansion being the addition of the chitin-protein layer on the epithelial side. It is presumably during expansion that the orientation occurs. The tube of the newly formed cuticle is initially under the uniform pressure of the epithelium, but as soon as buckling has occurred the axial restraint will be released and the expanding layer exposed to lateral restraint alone. After buckling the critical stress for lateral deformation will rise, the properties of the tube then being equivalent to a cylinder stiffened with supporting rings. As expansion proceeds the lateral pressure may therefore increase above the axial pressure which initiated buckling without inducing further deformation. This will culminate in the restraint imposed by the newly formed taenidia which eliminates some of the tubercles. Thus if at some stage during expansion the tracheal cuticle contains long, unoriented particles, their final orientation should be axial.

Histochemical changes in the trachea during its formation

According to Wigglesworth (1947) the newly secreted cuticulin layer over the abdomen in *Rhodnius* gives a strong positive reaction with Millon's reagent. This is much less obvious in the tracheae. Perhaps this is due to its extreme thinness, but it may also be correlated with the lack of pigment. The new trachea remains almost Millon-negative until the 12th day, when the taenidia are fully formed, after which it reacts positively. Most of the colour appears to be in the lining membrane, but there is some in the taenidia.

Changes in PAS staining are the reverse of those given by the Millon test. At 8 days, before the secretion of the new cuticle, the epithelium stains strongly. So does the ecdysial membrane, the newly formed cuticle, and the newly formed taenidia on the 9th–10th days. By 12 days the completed trachea

almost ceases to stain; its very pale pink colour is similar to that in intermoult tracheae. By 13 days the ecdysial membrane has disappeared and the old taenidia stain strongly as they dissolve in the moulting fluid. It is perhaps as a result of attack by the moulting fluid that the tracheal exuviae are PAS-positive.

In theory chitin should be PAS-positive (Pearse, 1954), but Richards (1951, 1952) and Wigglesworth (1956, personal communication) record that some material which certainly contains a polysaccharide may not stain. Richards suggests that the carbohydrate in insect cuticles may be patent or masked. This seems to be so with the chitin in the lining membrane and taenidia of tracheae. The incorporation of Millon-positive material is associated with the tracheae becoming PAS-negative. Now chitin fails to give a positive PAS test if the glycol groups are substituted (Pearse, 1954), and this is what might be expected in an intimate chitin-protein association. In agreement with this Wigglesworth (1956) records that the endocuticle in *Rhodnius* becomes PAS-positive when it is attacked by the moulting fluid. The endocuticle in *Rhodnius* exuviae is almost Millon-negative, in sharp contrast to the rest of the exuvium, and gives a positive PAS test.

The masking of chitin in tracheae is different from that described by Richards. The masking by sclerotization in the bee is probably comparable to that in the abdominal epicuticle of *Rhodnius*. In exuviae this layer contains chitin but is PAS-negative, becoming positive after extraction with mild alkali. It seems probable that there may be at least three processes which interfere with the PAS test—the incorporation of protein in the taenidia, the tanning of the chitin-protein layer in the lining membrane of the trachea, and the tanning with lipid impregnation in the bee and the abdominal epicuticle of *Rhodnius*.

The structure and formation of tracheoles

Tracheoles are readily teased from the tergites of larval *Rhodnius* for whole-mount electron microscope preparations. They are blindly ending tubes with an annular or helical pattern on the walls (fig. 1, M), which shows up as a raised ridge in carbon-shadowed preparations (fig. 1, F). In section the structure is seen to be very simple. The lining membrane appears uniform and sharply delineated (90–120 Å thick) like the cuticulin layer on the tracheae (fig. 1, H). There is no chitin-protein layer and the taenidia are small and indistinct. The tracheole is enveloped in the cell-membrane and there may be traces of an inner membrane surrounding the tube. Superficially a tracheole resembles a trachea with a different buckling frequency. This suggested that the expansion and buckling hypothesis might be extended to account for tracheole formation.

With this in mind a number of measurements of radius and taenidial frequency were made on preparations similar to fig. 1, M in an attempt to obtain graphs for tracheoles similar to those in fig. 6 for tracheae. The results were not encouraging. The calculated initial thickness was never less than

200 Å, whereas the final thickness observable in photographs is not more than 120 Å. Similar results were obtained from sections and other published figures of tracheoles. If the expansion and buckling hypothesis were to be retained, this could only be explained by assuming that the tracheoles had extended in length after their formation. This would be expected from Wigglesworth's work on tracheole migration. There was a further difficulty in applying the hypothesis to tracheole formation. In tracheae there is an epithelial layer, some part of which could act as a restraint to the expanding cuticle. In tracheoles with a lining membrane not greatly thinner no structure had been found which could act as a restraint apart from the tracheoblast cell-membrane.

Both these difficulties were resolved by sectioning tracheoles during their formation. There is much growth of the testis and its tracheal supply during the 4th–5th moult. Testis membranes were sectioned 7–12 days after feeding. On the 10th day tracheoles were seen in the stage shown in fig. 1, G, L. The lining looks much as a buckled membrane might be expected to look, and on its outside is a second unbuckled membrane. It seems plausible to suppose that this second sheath-membrane is acting as the restraint to expansion of the inner lining. Assuming that expansion has taken place with little increase in tube length, the initial radius of this tube may be estimated from the amplitude of the waves. Using this estimate (diameter increase $= 77\%$), the initial thickness calculated from expression (2) comes out to be about 46 Å. Therefore with a diameter increase of 77%, if expansion is accompanied by swelling, the final thickness should be about 82 Å. The measured thickness is about 85 Å. Several other preparations have a similar buckling frequency in this early stage of formation. These estimates are only approximate and few preparations appear as reliable as fig. 1, G, but a better agreement with the expansion and buckling theory could not be expected.

DISCUSSION

The ecdysial membrane

This membrane was first seen in the fully formed condition (fig. 1, I) and it was then thought that it might correspond to an endocuticle comparable to that laid down over the rest of the body after feeding (Zwicky and Wigglesworth, 1956). The positive PAS test supported this, since Wigglesworth (1956) records a strong positive PAS for endocuticle attacked by the moulting fluid. But its appearance and situation when newly formed suggest that it is the homologue of the ecdysial membrane containing chitin described by Passoneau and Williams (1953) and Richards (1955). The loss of the membrane in the moulting fluid and after treatments for purifying chitin contrasts with Richards's ecdysial membrane, which contains chitin and is not attacked by the moulting fluid. The longitudinal striations in the newly formed membrane are a puzzling feature which shows that a coherent membrane may be formed without the agency of a cell surface. Richards (1955) figures a pleated pattern in an ecdysial membrane from the 'face' of an exuvium of a *Cecropia*

moth pupa which has a pattern not unlike fig. 1, J, but this is in a mature ecdysial membrane. The similarities between the membrane in tracheae, the ecdysial membrane of the American authors and Wigglesworth's post-feeding cuticle over the *Rhodnius* abdomen, suggest that they may all be variants of the same thing. Whatever the nature and function of the ecdysial membrane, it is unlikely that it plays any part in determining tracheal structure for there is no ecdysial membrane in tracheae not formed round existing tracheae.

Buckling and micelle orientation

The precision with which a description in physical terms fits a biological event is the only criterion of validity, but a good fit does not establish a causal sequence. Nevertheless, the simplicity and regularity of the deformation in the tracheal cuticle has allowed the hypothesis of expansion and buckling to be verified in a way which would be extremely difficult for many of the surface cuticular patterns—although these may occur in the same way. For example, the stellate pattern on the abdomen of larval *Rhodnius* described by Wigglesworth (1947) may be imitated by causing a thin film of rubber on a glass surface to swell with xylene.

If the interpretations of tracheal and tracheole formation given here are correct, they present a remarkable example of organic design (Pantin, 1951). An identical 'trick' has been used to form tubes with similar functional requirements at two different levels of organization. This leads to a real distinction between tracheae and tracheoles, in terms of the thickness of the lining and hence the buckling frequency, the structure responsible for the restraint, and perhaps the mechanism of expansion.

The orientation of chitin micelles by stress is now well established in insects (Fraenkel and Rudall, 1947; Lees and Picken, 1945; Picken, Pryor, and Swann, 1947). The taenidia are another example of orientation which must take place away from a cell surface. In the development of lepidopteran scales the reverse procedure may occur (Picken, 1949). Here the growth of long fibrillar aggregates may cause the elongation of the scales. The sensory hairs over the abdomen of *Rhodnius* also contain chitin oriented in the axis and they are probably protected by a cuticulin layer. The possibility should be considered that it may have a role in their formation.

The mechanism of expansion

Very little can be said about the mechanism until more is known about the chemistry of the cuticle. In tracheoles, expansion is probably the result of swelling. In tracheae also the first-formed layer gets thicker, losing its distinctness on the epithelial side, its place being taken by the chitin-protein layer. On the other hand, it is an unusual sort of swelling which allows the inner cuticulin membrane of the trachea to appear much the same during and after expansion by up to 300%. It seems to be a property of this layer to maintain an approximately constant thickness; it is remarkably similar in all cuticles examined, the variable factor being the chitin-protein layer on the

epithelial side. The total thickness (360–570 Å) of the cuticle in a fully formed trachea is more than twice the observed or calculated initial thickness, so that something more complex occurs during tracheal formation than the swelling of a single membrane. The extra thickness may be due to the incorporation of Millon-positive material when the membrane is stabilized by tanning 12 days after feeding.

Cuticle terminology applied to tracheae

The epicuticle is normally defined as the outer non-chitinous part of the cuticle. This would restrict it to the cuticulin and morphologically outer layers excluding the chitin-protein layer, with which it is intimately associated in both tracheae and surface cuticle (Locke, 1957). This would seem to be an artificial definition. If the term epicuticle is to be useful, it should include the chitin-protein layer. Terminology is not important where the structure is well understood, but the separation of the cuticulin and chitin-protein layers may tend to obscure what may be the most fundamental feature in cuticle development, the orientation of micelles by the expanding cuticulin.

REFERENCES

CASTLE, E. S., 1937. J. cell. comp. Physiol., **10**, 113.
EDWARDS, G. A., and others, 1954. Rev. Brasil Ent., **2**, 97.
FRAENKEL, G., and RUDALL, K. M., 1947. Proc. Roy. Soc. B, **134**, 111.
KAYE, G. W. C., and LABY, T. H., 1948. *Physical and chemical constants.* London (Longmans).
KEISTER, M. L., 1948. J. Morph., **83**, 373.
LOCKE, M., 1957. Quart. J. micr. Sci., **98**, 487.
LEES, A. D., and PICKEN, L. E. R., 1945. Proc. Roy. Soc. B, **132**, 396.
MITCHISON, J. M., and SWANN, M. M., 1954. J. exp. Biol., **31**, 443.
PANTIN, C. F. A., 1951. Advanc. Sci., **8**, 138.
PASSONEAU, J. V., and WILLIAMS, C. M., 1953. J. exp. Biol., **30**, 545.
PEARSE, A. G. E., 1954. *Histochemistry, theoretical and applied.* London (Churchill).
PICKEN, L. E. R., 1949. Phil. Trans. Roy. Soc. B, **234**, 1.
—— PRYOR, M. G. M., and SWANN, M. M., 1947. Nature, **159**, 434.
RICHARDS, A. G., 1951. *The integument of Arthropods.* Minneapolis (University of Minnesota Press).
—— 1952. Science, **115**, 206.
—— 1955. J. Morph., **96**, 527.
—— and ANDERSON, T. F., 1942. J. N.Y. Ent. Soc., **50**, 147.
—— and KORDA, F. H., 1950. Ann. Ent. Soc. Amer., **43**, 49.
TACHIBANA, T., INOKUCHI, K., and INOKUCHI, T., 1955. Nature, **176**, 1117.
THOMPSON, W. R., 1929. Trans. Roy. Ent. Soc. Lond., **77**, 195.
TIMOSHENKO, S., 1936. *Theory of elastic stability.* New York (McGraw-Hill).
WIGGLESWORTH, V. B., 1931. Biol. Rev., **6**, 181.

WIGGLESWORTH, V. B., 1933. Quart. J. micr. Sci., **76**, 269.
—— 1934. Ibid., **77**, 191.
—— 1947. Proc. Roy. Soc. Lond. B, **134**, 163.
—— 1954. Quart. J. micr. Soc., **95**, 115.
—— 1956. Ibid., **97**, 89.
WOLFE, L. S., 1954. Ibid., **95**, 49.
ZWICKY, K. T., and WIGGLESWORTH, V. B., 1956. Proc. Roy. Ent. Soc. Lond., A, **31**, 153.

The Coelomocytes of *Holothuria leucospilota*

By R. ENDEAN

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With one plate (fig. 4)

SUMMARY

Morula-shaped cells, structurally similar to fibre-producing cells occurring in the blood and test of an ascidian, *Pyura stolonifera*, have been found in the coelomic fluid and Cuvierian tubules of *Holothuria leucospilota*.

The morula-shaped cells of the holothurian are formed from primitive amoebocytes. They contain globules, each of which consists of a central core of proteinaceous material associated with an unidentified iron compound. A film of polysaccharide material, containing granules of a lipo-protein nature, surrounds the core. Part, at least, of the polysaccharide is mucopolysaccharide. It is believed that the materials present in the globules are precursors of collagenous connective tissue fibres.

The mean value of the iron content of the corpuscles was found to be 0·12 g per 100 g dry weight. The iron is organically bound but does not appear to have chemical relationship to haem or other similar iron-porphyrin compounds.

Fibrous processes arise from many morula-shaped cells when they are cytolysed with distilled water.

In hanging-drop preparations some morula-shaped cells eject globules which trail fibres. It is believed that the globules are directly involved in fibre-production.

Granules, similar in many respects to the globules of the morula-shaped cells of the holothurian, have been found in vertebrate fibroblasts.

INTRODUCTION

A VARIETY of free cells, known generally as coelomocytes, occurs in the coelomic fluids, haemal systems, and water-vascular systems, and amongst the tissues of holothurians. These cells have been investigated by numerous workers and the results of these investigations are well summarized by Hyman (1955). Although the structure and distribution of each of the various cell-types have received considerable attention, little information is available on the chemical constitution and role of each.

The present paper is concerned with investigations made on the coelomocytes of *Holothuria leucospilota* Brandt. Attention is focused on aspects of their structure and chemical constitution which might have a bearing on the biosynthesis and deposition of collagen fibres within the Cuvierian tubules.

MATERIAL AND METHODS

Specimens of *H. leucospilota* were obtained, initially, from Heron Island, in the Capricorn Group, Queensland, and subsequently from Caloundra, southern Queensland.

Coelomic fluid surrounds the organs lying in the body-cavity. The fluid was obtained for study as follows. A specimen was picked up and ejection of its Cuvierian organs prevented by placing a spring-loaded steel paper-clip

over its anal end. Grit and sea-water adhering to the animal were removed with a cloth and a longitudinal incision made through the body-wall, in an oral direction from a point midway between oral and anal ends of the animal. The coelomic fluid which ran from this incision was collected in a beaker. If the whole operation was carried out rapidly, the procedure followed minimized the chances of the cloacal wall rupturing and of the exposure of the sticky Cuvierian tubules. Adult specimens of average size yielded 50 to 80 ml of cloudy coelomic fluid.

Cuvierian tubules and portions of the stem of the respiratory trees were removed from specimens and fixed variously with Bouin's fluid, Heidenhain's 'Susa', and 5% formalin in sea-water. Sections were stained with Mallory's aniline blue collagen stain and with Heidenhain's 'Azan'.

The Coelomocytes

Coelomocytes present in the coelomic fluid showed a marked tendency to clump together when coelomic fluid was withdrawn from the body of the

2μ

FIG. 1. Morula-shaped cell.

holothurian and this vitiated attempts to make accurate counts of coelomocyte numbers by the use of a haemocytometer. Centrifugation of the coelomic fluid yielded a greyish mass of coelomocytes. The average volume occupied by coelomocytes from 10 ml of coelomic fluid which had been centrifuged at 3,000 r.p.m. for 5 min was 0·08 ml.

Morula-shaped cells. Morula-shaped cells (fig. 1) usually comprised about 70% of the total number of coelomocytes found in the coelomic fluid. These cells appeared to correspond with the 'migratory plasma cells' of Hamann (1883), the 'cellules muriformes' of Hérouard (1889) and of Cuénot (1891), and the 'colourless amoebocytes with spherules' of Théel (1921) and of Hyman (1955). They also bore a striking resemblance to the 'ferrocytes' of the ascidian, *Pyura stolonifera* (Heller) (Endean, 1955a).

The morula-shaped cells were colourless and spherical at rest. They ranged from 8 to 16 μ in diameter. Each contained a variable number of refractile globules encased in cytoplasm. In their natural position the globules were mutually compressed, but if the cells were cytolysed with distilled water, the globules that burst forth were spherical. The globules averaged about 1 μ in diameter, but in the larger cells many were as large as 2 μ.

Observations with the phase-contrast microscope on cytolysing cells revealed that the emitted globules were accompanied by a granulated viscous material which apparently surrounded them in the intact cell. An eccentrically placed nucleus, about 2·5 μ in diameter, was also revealed; this was obscured in life by the globules.

The larger morula-shaped cells (13 to 16 μ in diameter) were amoeboid. Blunt pseudopods were put out which extended 2 to 3 μ from the cells. Into these pseudopods the globules eventually flowed, rolling over one another in the process. The smaller morula-shaped cells did not exhibit such activity and were spherical.

With neutral red (0·0001% in sea-water), the smaller non-amoeboid cells stained bright red whilst the larger ones stained brownish red. In both cases when cells so stained were cytolysed with distilled water the globules that emerged were colourless. It would appear that it is the granulated material surrounding them in the intact cell which takes up the stain.

With methylene blue (0·0001% in sea-water), the smaller cells stained greenish blue and the larger ones purplish. When cells so stained were cytolysed with distilled water the globules that emerged in both cases were stained a pale blue. It is believed that the granulated fluid normally surrounding the globules is responsible for the metachromasy exhibited. The nuclei of the cells became evident when the obscuring globules were released by cytolysis and these nuclei stained faintly with methylene blue. It was noted that the nucleus was relatively larger in the smaller cells than in the larger ones.

Methyl red indicator was added to a drop of coelomic fluid but it did not penetrate into the interior of any of the coelomocytes.

Morula-shaped cells, identical with those found in the coelomic fluid, occurred in abundance amongst the fibres of the connective tissue in the walls of the respiratory trees and papillae from which the Cuvierian tubules arise. Similar cells occurred also in the central core of the tubules and amongst the fibres present in the tubules. In the tubules, however, the morula-shaped cells were usually large amoeboid ones. (See Endean, 1957.)

The morula-shaped cells appeared to enter the core of each tubule by migrating from the connective tissue of the papilla.

Homogeneous amoebocytes. These cells were from 4 to 5 μ in diameter and consisted essentially of a large nucleus (3·5 to 4 μ in diameter) containing many chromatin granules and invested by a thin cytoplasmic envelope (fig. 2, A). Usually these cells were spherical but some possessed filamentous pseudopodia (fig. 2, B).

These cells seem to be the primitive cells in holothurian coelomic fluid and

they may give rise to other cell types. Transitional stages from homogeneous amoebocyte to morula-shaped cell were commonly found. This transition seemed to occur as follows:

(1) Numerous minute vacuoles appeared in the cytoplasm of a homogeneous amoebocyte (fig. 2, C). These vacuoles stained blue with dilute methylene
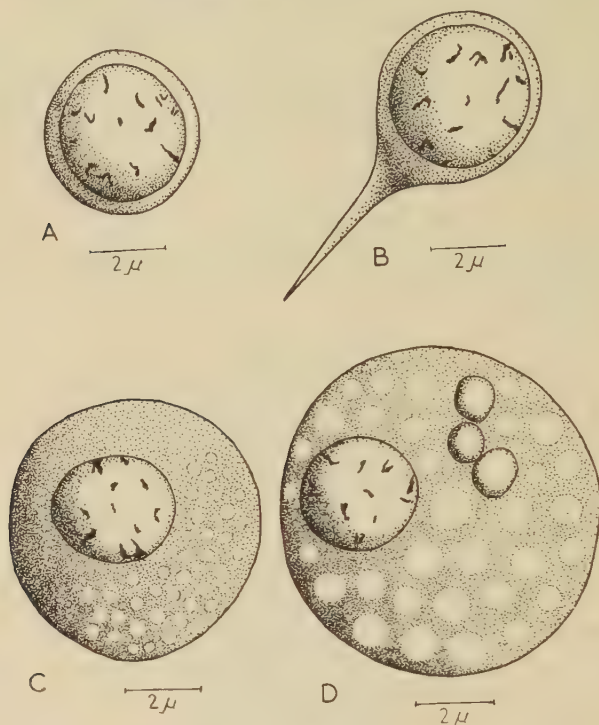
FIG. 2. A, homogeneous amoebocyte. B, homogeneous amoebocyte with filamentous pseudopodium. C, amoebocyte with vacuolated cytoplasm. An early stage in the transition from homogeneous amoebocyte to morula-shaped cell. D, amoebocyte with vacuoles and globules. A later stage in the transition from homogeneous amoebocyte to morula-shaped cell.

blue but did not stain with dilute neutral red. (In some cases two or three small bodies which stained with neutral red (possibly nucleoli) were observed against the nucleus.)

(2) The vacuoles enlarged and there was a concomitant increase in the size of the cell containing them.

(3) The vacuoles continued to enlarge and each gradually acquired a peripheral layer of refractile material which stained red with neutral red (fig. 2, D).

(4) By the time each vacuole had acquired a refractile layer the cells had attained a diameter of 8μ and were identical with the smaller morula-shaped cells.

The nucleus becomes smaller during these transition stages and it is possible that nuclear material is utilized in the formation of the globules.

Homogeneous amoebocytes appeared to be formed in the epithelium of the lumen of the respiratory trees, where they occurred in abundance. There was a general tendency for the average size of amoebocytes present in the walls of the respiratory trees to increase from the lumen outwards towards the periphery. This may indicate that there is an outward migration of homogeneous amoebocytes from their sites of origin in the epithelium of the lumen to the
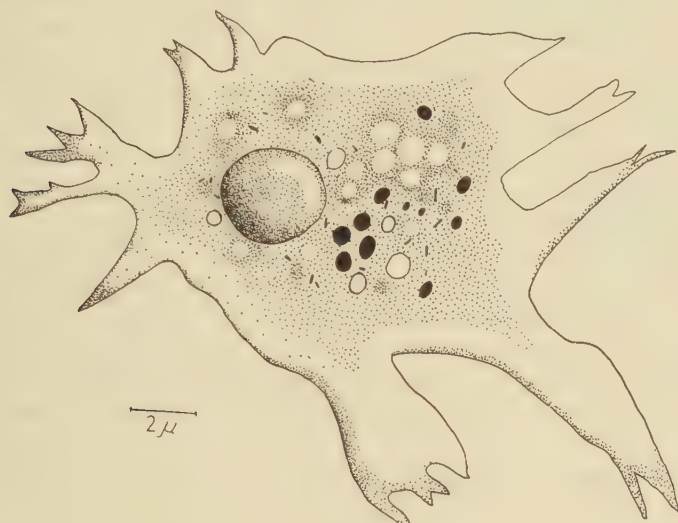
FIG. 3. Phagocyte.

coelomic fluid. Should this be the case, it would be expected that there would be a continual influx of homogeneous amoebocytes into the tubules from the epithelium of extensions of the lumen present in the papillae. Apparently there is such an influx because numerous homogeneous amoebocytes (recognized by their nuclear diameter, poverty of cytoplasm, and staining reactions, and by their protrusion of filamentous pseudopodia) occurred amongst the fibres present in the tubules.

Amoebocytes, presumably migrating to the coelomic fluid, were observed in the coelomic lining of the respiratory trees. Also bodies about 4μ in diameter were observed in the cells of the outer layer of the tubules; these stained with nuclear stains. It was not possible to ascertain whether these bodies were amoebocytes or whether they were the nuclei of the cells of the outer layer. Since some cells possessed more than one of these bodies it is thought probable that some, at least, were homogeneous amoebocytes migrating through these cells to the coelomic fluid.

Phagocytes. Approximately 10% of the total number of coelomocytes were large cells (fig. 3), possessing irregular outlines. Long pseudopods radiated from these cells, which contained a small nucleus, granules, vacuoles and a

varying number of solid bodies of different sizes. These bodies had irregular shapes and were probably ingested material—in some cases ingested morula-shaped cells. It is for this reason that the cells are believed to be phagocytic. Some of the vacuoles stained with dilute methylene blue.

Phagocytes were not observed in the wall of the respiratory trees, in the papillae, or in the tubules.

Spindle cells. These cells were spindle-shaped and each ranged from 6 to 10 μ in length. They comprised about 10% of the total number of coelomo-cytes and each possessed a refractive outer cytoplasm which rendered observation of internal structures difficult. Their cytoplasm stained faintly blue with dilute methylene blue and this stain showed the presence in each of a large nucleus. Dilute neutral red revealed the presence of a variable number of small granules in the cytoplasm.

The function of these cells is not known. They were not found in the tubules or in the walls of the respiratory trees or papillae. Similar cells were found in the genus *Molpadia* by Ohuye (1936).

Crystal cells. About 5% of the cells observed in the coelomic fluid were each filled with a rhomboidal crystal. These cells were 9 to 11 μ long, 7 to 9 μ wide, and 4 to 5 μ thick. In surface view no cytoplasm was evident, but in side view a thin film could be seen. In a few cells two rhomboidal crystals were united. These cells did not stain with either dilute methylene blue or dilute neutral red. They were not observed in the tubules or walls of the respiratory trees or papillae.

Similar cells have been found in other species of holothurians by Théel (1921).

HISTOCHEMISTRY OF THE MORULA-SHAPED CELLS

Drops of coelomic fluid were placed on slides and a drop of 5% formalin in sea-water added to each. In each case a coagulum was formed which settled on the slides. The drops were then allowed to stand for about 10 min and fluid was then washed from the slides with distilled water. Many cells were lost as a result of this procedure and many were cytolysed, but a proportion remained intact and were suitably fixed.

Coelomocytes treated as above were stained with iron haematoxylin. The nuclei of all cells stained heavily and chromatin granules were evident. The globules of the morula-shaped cells and the vacuoles of their precursors stained blue-black.

With Heidenhain's Azan the globules in the smaller morula-shaped cells and those released from both large and small cytolysed cells stained blue. However, the globules of many of the larger intact morula-shaped cells stained red. All gradations in intensity of staining from a deep blue to a deep red were observed. Sometimes within the same cell some globules stained blue and others red.

The reasons for such staining are obscure but again evidence is presented that the larger globules consist of two components.

Formalin-fixed morula-shaped cells were stained for polysaccharides by Hotchkiss's (1948) method. Intact cells gave a strong positive reaction, as did the granulated material from cytolysed cells. The globules themselves, when released, stained only faintly. Intense metachromasy with toluidine blue was exhibited by the globules of the larger formalin-fixed morula-shaped cells, but the globules of smaller cells stained a pale blue or not at all. In the case of the larger cells it was found that the globules, when free, also stained a pale blue and the metachromatic staining observed was given by the granulated material which surrounds the globules in intact cells but which forms a granulated coagulum under the action of formalin when released from these cells.

Since the results of the above tests indicated the presence of highly polymerized polysaccharides it was thought that part, at least, of these might be acid mucopolysaccharides. These substances were sought by using Hale's (1946) histochemical method, which requires fixation in Carnoy's fluid. When a drop of this fixative was added to a drop of coelomic fluid a coagulum was formed and all the coelomocytes present were cytolysed. However, clumps of cytolysed cells stained strongly for acid mucopolysaccharides.

Formalin-fixed morula-shaped cells darkened slightly with Sudan black B. It was established that the granules in exudates of the viscous material which formed films around the globules took up the colouring agent and possibly contained a small quantity of neutral lipid.

Formalin-fixed cells were next subjected to the mercuric chloride bromphenol blue technique of Mazia, Brewer, and Alfert (1953) for the histochemical staining of protein. The morula-shaped cells became dark blue, indicating that proteins were present. Free globules from morula-shaped cells stained heavily, indicating the presence of considerable quantities of protein. The granules in exudates from cytolysed cells of the viscous material which forms films around the globules also stained for proteins, but the viscous material itself stained only faintly, if at all.

Because of the similarity between the morula-shaped cells of *H. leucospilota* and the ferrocytes of *Pyura stolonifera* it was thought that iron might be present. Inorganic iron was first sought by using the Prussian blue reaction and Humphrey's (1935) dinitrosoresorcinol test. Iron was not detected. However, when formalin-fixed cells and alcohol-fixed cells were placed in 3% nitric acid at 35° C for 24 h and then stained for iron, positive results were obtained both with ferrocyanide in hydrochloric acid and with dinitrosoresorcinol. Also it was ascertained that the organically bound iron was confined to the globules and little if any appeared to be present in the viscous material surrounding them.

The histochemical tests described above indicate that the globules of the morula-shaped corpuscles each contain an inner core of protein associated with an iron compound. The iron compound is surrounded by a film of polysaccharide, part or all of which is possibly in the form of acid mucopolysaccharide, and in this film granules containing lipid and protein are found.

THE IRON COMPOUND

Since organically bound iron had been detected histochemically in the globules of the morula-shaped coelomocytes, attempts were made to detect it in coelomocytes removed from the coelomic fluid by centrifugation. Care was taken to prevent cytolysis of the coelomocytes from occurring. Samples, each consisting of the coelomocytes from 20 ml of blood, were used.

Four samples were each heated with 10 ml of glass-distilled water to which 2·5 ml of concentrated nitric acid and 1 ml of concentrated sulphuric acid were added, until all trace of organic material was removed. The samples were then neutralized with NaOH. Two of them were tested for iron by Sandell's (1944) *o*-phenanthroline technique. A reddish colour was obtained, indicating the presence of iron. Confirmation of this finding was obtained by acidifying the other two samples with nitric acid and adding potassium ferrocyanide solution. Precipitates of Prussian blue were obtained.

The concentration of iron in the coelomocytes from 20 ml samples of coelomic fluid taken from 7 different specimens was determined by the method of Sandell (1944). The mean value for the iron content of the coelomocytes was found to be 0·12 g per 100 g dry weight of the coelomocytes, with a range of 0·1 to 0·13 g per 100 g dry weight.

Haemoglobin has been found in the coelomocytes of several species of holothurians (Howell, 1885; Kollman, 1908; Hogben and van der Lingen, 1928; Kobayashi, 1932). Hyman (1955, p. 147) stated that haemoglobin-containing cells ('hemocytes') were 'found throughout the class'. However, cells answering the description of these hemocytes were not observed in *H. leucospilota*. Nevertheless, there is the possibility that the iron compound in the morula-shaped coelomocytes of this species is haemoglobin. Examination with a microspectroscope of the coelomic fluid, centrifuged coelomocytes, and suspensions of coelomocytes cytolysed with distilled water, failed to reveal any absorption bands.

The iron compound in the morula-shaped coelomocytes of *H. leucospilota* shows marked resemblances to the iron compound present in the ferrocytes of the ascidian, *Pyura stolonifera*. In both cases the compound is present in structurally similar morula-shaped cells at about the same concentrations. In neither case does it appear to have chemical relationship to haem or other similar iron-porphyrin compounds.

In view of this it was decided to investigate other aspects of the chemical constitution of the morula-shaped coelomocytes and to ascertain whether further similarities existed between them and the ferrocytes. In particular, it was decided to investigate the intracellular acidity of the morula-shaped cells and also the reducing properties of the intracellular material.

INTRACELLULAR ACIDITY OF THE COELOMOCYTES

The coelomic fluid, as determined by the glass electrode, showed a constant pH of 7·7 and was therefore less alkaline than sea-water. When the coelomocytes in 10 ml samples of the coelomic fluid were cytolysed by freezing,
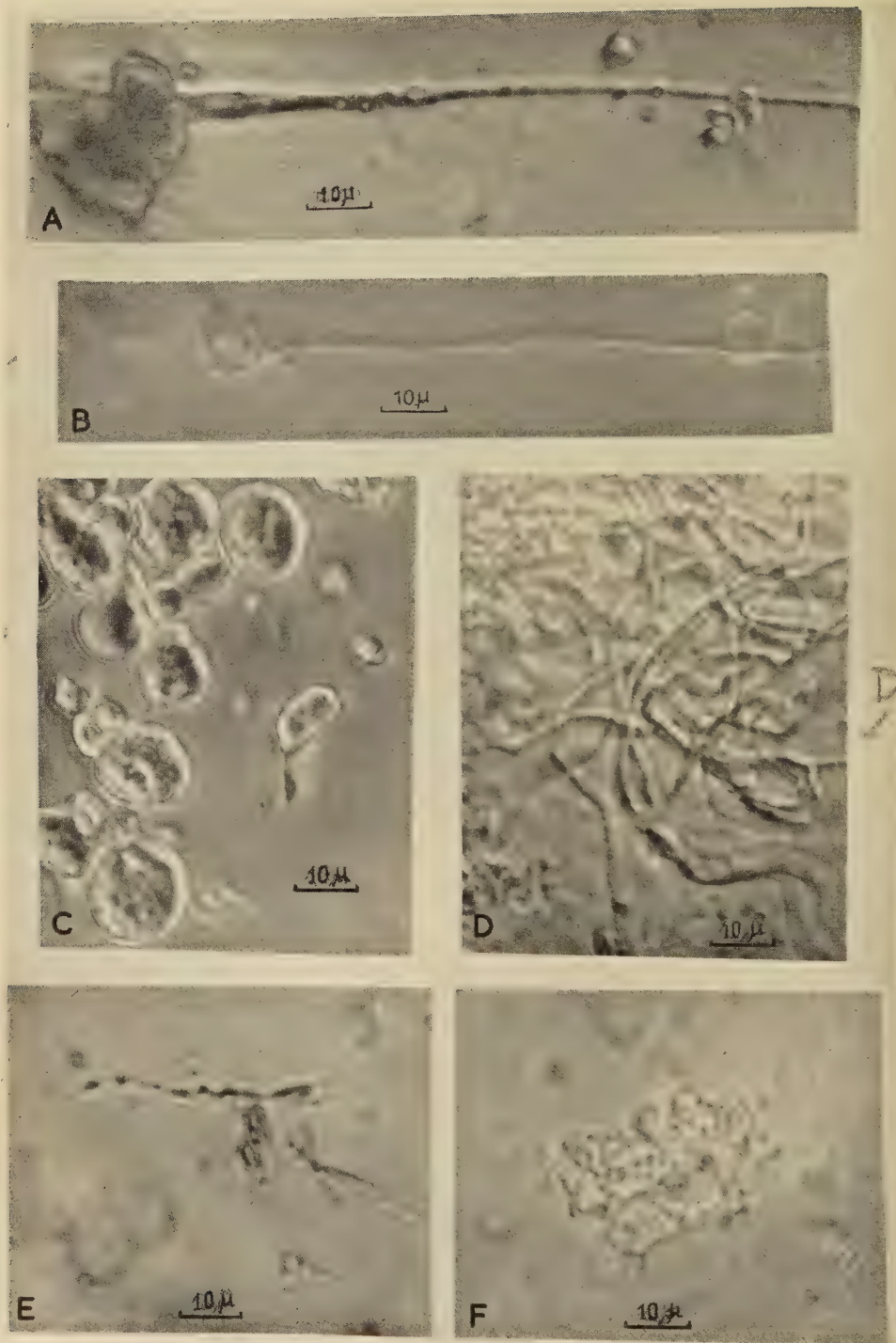
Fig. 4

R. ENDEAN

the pH of the fluid fell to 7·2–7·4. Possibly the buffering power of the coelomic fluid affected this result. Therefore, the coelomocytes in 10 ml samples of coelomic fluid were centrifuged off, and the clear fluid above them was poured off and replaced by equivalent amounts of glass-distilled water. The pH of the resulting mixtures in each case lay between 6·5 and 6·7. It would seem that a small amount of acid is present in the coelomocytes.

REDUCING PROPERTIES OF THE INTRACELLULAR MATERIAL OF THE COELOMOCYTES

Samples of coelomocytes centrifuged off in each case from 10 ml of coelomic fluid were each cytolysed with 5 ml of glass-distilled water. The intracellular material did not reduce dilute potassium permanganate or potassium dichromate solutions.

FIBRE FORMATION

Fibre production by coelomocytes. When 10% formalin or 96% alcohol was added to drops of coelomic fluid placed on slides, the coelomocytes cytolysed and a coagulum in which filaments were apparent was formed (fig. 4, A). The filaments enmeshed the cytolysed coelomocytes and in many cases appeared to have arisen from them. Adjacent clumps of cytolysed coelomocytes were usually linked by straight filaments which in places were sufficiently thick to form a film.

The above phenomena have a parallel in observations made by Théel (1921) on the coelomic fluid of *Mesothuria intestinalis* (Ascanius and Rathke). A coagulum similar to that described above was formed in the coelomic fluid of this species when the fluid was placed in a glass tube. Théel (p. 26) believed that there was 'an abundance of fibrin matter in the coelomic fluid' and that 'plasma amoebocytes' were not present in sufficient numbers to play a major part in the process.

The nature and significance of the coagulum formed by the coelomic fluid of *H. leucospilota* when treated with alcohol or formalin are not known. However, it was observed that when coelomocytes separated from the coelomic fluid by centrifugation were cytolysed with distilled water, what appeared to be fibrous processes arose from many of the morula-shaped coelomocytes

FIG. 4 (plate). A, photomicrograph of a fibrous process formed from a clump of morula-shaped cells as a result of fixation with 5% formalin in sea-water. Stained with Hotchkiss's polysaccharide stain.

B, photomicrograph of a fibre which has arisen from a morula-shaped cell cytolysed with distilled water. Phase contrast.

C, photomicrograph of a cluster of globules linked by a thread to a morula-shaped cell from which they have been ejected. The cell has been kept in a hanging drop preparation and was photographed 2 h after removal from the holothurian. Phase contrast.

D, photomicrograph of 'beaded' fibres formed from cells which had cytolysed on standing.

E, photomicrograph of globules which had been ejected from a morula-shaped cell kept for 24 h in a hanging drop preparation. The globules are linked together by fibres. Phase contrast.

F, photomicrograph of fibres formed from globules ejected from a morula-shaped cell kept for 24 h in a hanging drop preparation. Globules are still evident but many of these have decreased in size. Phase contrast.

(fig. 4, B). Similar structures did not form in the coelomic fluid from which the coelomocytes were removed. It would seem therefore that the fibrous processes are formed primarily from material present in the coelomocytes.

When coelomocytes, concentrated by centrifugation, were left to stand in a little coelomic fluid, in many cases definite fibres arose from small clumps of cells which had cytolysed and sometimes such fibres arose from single morula-shaped cells. All the fibres, however, had a beaded appearance (fig. 4, D). The cells and fibres were placed on slides and fixed with 5% formalin in sea-water. The 'beads' stained positively for polysaccharides with Hotchkiss's (1948) technique and they also exhibited metachromasy with toluidine blue. In the case of the fibres themselves negative results were obtained, but they stained positively though faintly for protein when the histochemical method of Mazia, Brewer, and Alfert (1953) was used. It is believed that the 'beads' are globules and that these are linked together by protein fibres.

Hanging drop preparations of coelomocytes were examined at regular intervals. In a few cases short fibres were formed from morula-shaped coelomocytes. The mode of formation was as follows. A projection appeared on the surface of a morula-shaped coelomocyte. In this projection granules showing violent movement were noted and from the projection one or more globules were hurled out, sometimes as far as $16\,\mu$ from the cell. The globule or globules were attached by slender fibres to the cell which gave rise to them (fig. 4, C). Other globules accompanied by granules moved out of the cell and passed along the fibres. Sometimes numerous globules arranged themselves like beads along the length of the fibre. The appearance of such fibres is identical with that of the beaded fibres mentioned earlier.

Frequently one or more globules, after moving back and forth along a fibre, would suddenly shoot off in a new direction, each trailing a fibre. Globules which gave rise to such fibres decreased in size and presumably intraglobular material is utilized in the formation of the fibres. Often the globules collided with one another whilst moving back and forth along the fibres. When such a collision occurred the colliding globules appeared to merge temporarily but soon snapped apart from one another (fig. 4, E).

Small meshworks of fibres were sometimes formed but the fibres were never very long and were oriented in all directions. Usually only short fibres were formed from a single morula-shaped cell (fig. 4, F).

Fibre production within the Cuvierian tubules. A satisfactory procedure for observing fibre production in position within the Cuvierian tubules has not yet been devised.

Study of fresh whole mounts of tubules revealed an abundance of morula-shaped coelomocytes within the central core of each tubule. Coelomocytes of the same type were also present between the fibres of the tubule connective tissue, but these coelomocytes were in all stages of apparent degeneration. Often the globules had broken free from the cells and frequently such globules were disposed in lines between the fibres.

Sections through the tubules showed that such globules were almost

lways connected by fibrous processes to the cells from which they were
erived and in many cases similar fibrous processes radiated out from the
norula-shaped cells themselves and from the emitted globules. However,
ecause of the uncertain action on the morula-shaped cells of the fixatives
sed (Bouin's fluid and Susa), it is not possible to equate these fibrous pro-
esses with true fibres.

DISCUSSION

Reflection upon the histology and histochemistry of the Cuvierian tubules
nd coelomocytes of *H. leucospilota* will reveal that, of the cells present in the
ubules, only the morula-shaped coelomocytes could be involved, directly,
n the synthesis of fibrillar material and in the deposition of fibres. The
vailable evidence indicates that the globules of these cells contain materials
vhich are probably precursors of the collagenous connective tissue of the
ubules.

It would seem that amoebocytes, formed in the epithelium lining the lumen
f the respiratory trees, develop in their cytoplasm vacuoles in which pro-
einaceous materials are found. The vacuoles enlarge and each gradually
cquires a peripheral film of mucopolysaccharide. Subsequently, in some cases
t least, these globules pass out of the morula-shaped cells so formed into
he interfibrillar substance of the tubules. Here their contained protein and
nucopolysaccharide may be utilized in the formation of collagen fibres.

'Ferrocytes' that participate in the formation of fibres and are similar in
hape to the holothurian morula-shaped cells have been found in the ascidian
Pyura stolonifera (Endean, 1955a, 1955b, 1955c). A comparison of the pro-
erties possessed by the ferrocytes and holothurian morula-shaped cells is
nstructive (Table 1).

It can be seen that the morula-shaped coelomocytes of the holothurian and
he ferrocytes of the ascidian are similar in many respects. Thus both are
tructurally similar, both are formed in a comparable fashion, and both can
roduce fibres. Both contain an acid polysaccharide which appears to be a
nucopolysaccharide and both contain unidentified iron compounds which do
ot appear to have chemical relationship with haem or other familiar iron-
orphyrin compounds. It is possible that the iron compounds have roles in
he polymerization processes which must occur within the globules of both
ells. The chemical constitution of these iron compounds will be discussed in
. later paper.

The differences exhibited by the morula-shaped cells of the holothurian
nd the ferrocytes of the ascidian are of importance. In particular, the obser-
ations that the intra-globular material of the holothurian morula-shaped cells
vas proteinaceous whilst the intraglobular material of the ferrocytes appeared
o be polysaccharide are of interest because the former cells are involved in the
ormation of a structural protein (collagen) whilst the latter are concerned with
he production of a structural polysaccharide (tunicin). Other major differences
oncern the acidity and reducing power of the intracellular material. In the

TABLE I

Property investigated	Ferrocytes of Pyura stolonifera	Morula-shaped coelomocytes of H. leucospilota
Shape	Morula-shaped	Morula-shaped
Average diameter	8μ	14μ
General structure	Globules (12–38) encased in colourless cytoplasm	Globules (18–90) encased in colourless cytoplasm
Movement	Blunt pseudopodia	Blunt pseudopodia
Supravital staining with neutral red	Globules stain. Those of non-amoeboid cells stain most strongly. (Not noted whether staining is confined to layer at periphery of each globule)	Globules stain. Those of non-amoeboid cells stain most strongly. Staining confined to layer at periphery of each globule
Supravital staining with methylene blue	Globules stain	Globules stain
Possession by globules of refractile layer	Globules possess refractile layer	Globules possess refractile layer
Intracellular acidity	Strongly acid	Faintly acid
Reducing properties of intra-cellular fluid	Reduces $KMnO_4$ and $K_2Cr_2O_7$	Does not reduce $KMnO_4$ or $K_2Cr_2O_7$
Presence of iron compound	Iron compound present	Iron compound present
Colouring with Sudan black B	None observed	Globules coloured faintly
Histochemical staining for polysaccharide	Globules and intra-globular material from cytolysed globules stained strongly	Globules stained strongly but staining confined to film on periphery of each globule
Staining with toluidine blue	Globules stained red; not noted whether metachromatic staining confined to surface film	Globules stained red but staining confined to film on periphery of each globule
Histochemical staining for protein	Cells stained strongly but not noted whether globules stained or whether staining was confined to protoplasm surrounding globules	Cells stained and globules stained strongly
Action of distilled water	Cells cytolysed and globules liberated. When globules burst slime is formed. Condensations in slime give rise to fibres. Granules present along length of fibres	Cells cytolysed and globules liberated. Fibrous processes arose directly from morula-shaped cells
Behaviour in hanging drop suspensions	A minority of cells put out thread-like protoplasmic extensions with a terminal knob. In a few cases thick fibres arose from terminal knob	In a few cases a globule (or globules) was shot out of a morula-shaped cell. The globule (or globules) was attached by a fibre to the cell. Other globules accompanied by granules moved out of the cell and passed along the fibres. These globules often formed additional fibres
Mode of formation	From primitive lymphocytes. Vacuoles precede globules. A signet-ring stage	From primitive amoebocytes. Vacuoles precede globules. No signet-ring stage

ferrocytes this material is strongly acid and has marked reducing power whilst in the coelomocytes of the holothurian the intracellular material appears to be only faintly acid and does not show similar reducing properties. The manner in which these differences are related to the synthesis of the precursors of tunicin and collagen, if indeed they are, is not known.

In fibrogenic cells of the fowl embryo grown in serum exudate, conspicuous granules ranging from 0·7 to 2 μ in diameter have been found (Jackson and Smith, 1955). These granules are therefore similar in size to the globules contained in the morula-shaped cells of *H. leucospilota*. Moreover, both granules and globules stain supravitally with neutral red, exhibit metachromasy with toluidine blue, and stain histochemically for polysaccharide and acid polysaccharide. In both cases part at least of the polysaccharide is a mucopolysaccharide. Also both granules and globules contain protein.

It was noted by Jackson and Smith (1955) that the centres of the granules found in the fibroblasts of the embryonic chick were less dense than the periphery and that 'even after prolonged treatment with periodic acid and Schiff's reagent only the periphery of the granules stain while the centres remain colourless' (p. 92). In each globule of the morula-shaped cells of the holothurian studied, a central proteinaceous area is surrounded by a film of acid polysaccharide material. It is intended to ascertain whether the protein and polysaccharide in the granules of the fibroblasts of the developing fowl are similarly distributed and also whether the protein is associated with an iron compound as it is in the morula-shaped cells.

Both granules and globules, when removed from the cells where they are formed, show a tendency to line up and form fibres with a beaded appearance. The chemical nature of the beaded fibres formed by the isolated granules was not stated but each of those formed by the globules of the morula-shaped cells of *H. leucospilota* consists of globules united by a protein thread.

No normal extrusion of intact globules was observed in the case of the fibroblasts of the developing fowl. By analogy with the mode of formation of fibres from rabbit fibroblasts (Stearns, 1940) it might be expected that protoplasmic protruberances would eventually arise from these fibroblasts and that in the vicinity of these protruberances fibres would form.

Globules were observed to be thrown out of the morula-shaped cells of *H. leucospilota*. These globules were linked by fibres to the cell and it would appear that fibres can arise directly from the intraglobular material. Whether fibres are normally formed in this fashion inside the Cuvierian tubules is not known, but it may be significant that globules are usually released from morula-shaped cells occurring amongst the collagenous fibres of the tubules.

of the author's drawings were prepared by Mr. E. Hollywood of the Photographic Department, University of Queensland.

REFERENCES

CUÉNOT, L., 1891. Arch. Biol., **11**, 313.

ENDEAN, R., 1955a. Aust. J. Mar. Freshw. Res., **6**, 35.

—— 1955b. Ibid., **6**, 139.

—— 1955c. Ibid., **6**, 157.

—— 1957. Quart. J. micr. Sci., **98**, 455.

HALE, C. W., 1946. Nature, **157**, 802.

HAMANN, O., 1883. Z. wiss. Zool., **39**, 145.

HÉROUARD, E., 1889. Arch. Zool. exp. gén., **7**, 535.

HOGBEN, L., and VAN DER LINGEN, J., 1928. J. exp. Biol., **5**, 292.

HOTCHKISS, R. D., 1948. Arch. Biochem., **16**, 131.

HOWELL, W. H., 1885. Johns Hopk. Univ. Circ., **5**, 5.

HUMPHREY, A. A., 1935. Arch. Path. (Lab. Med.), **20**, 256.

HYMAN, L. H., 1955. *The invertebrates: IV. Echinodermata.* New York (McGraw-Hill).

JACKSON, S. FITTON, and SMITH, R. H., 1955. Symp. Soc. exp. Biol., **9**, 89.

KOBAYASHI, S., 1932. Sci. Rep. Tôhoku Univ., **7**, 211.

KOLLMAN, M., 1908. Ann. Sci. Nat. Zool., **8**, 1.

MAZIA, D., BREWER, P. A., and ALFERT, M., 1953. Biol. Bull., **104**, 57.

OHUYE, T., 1936. Sci. Rep. Tôhoku Univ., **11**, 207.

SANDELL, E. B., 1944. *Colorimetric determination of traces of metals.* New York (Interscience Publishers).

STEARNS, M. L., 1940. Amer. J. Anat., **67**, 70.

THÉEL, H., 1921. Arkiv. Zoologi, **13**, No. 25.

A Technique for Marking the Site of Recording with Capillary Micro-electrodes

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With one plate (fig. 1)

SUMMARY

Records were made from the hind-brain of 15-cm tench (*Tinca tinca*, Osteichthyes) by the use of capillary micro-electrodes. At the completion of recording the electrodes were left in position and the site of recording was marked by the following method. The Ringer bathing the brain was replaced by a mixture of equal parts of 110 mM $FeCl_3$ and Ringer. After 4 h, ferrocyanide ions were released electrolytically from the micro-electrode. The electrolyte in the micro-electrodes was 2·5 M $KCl+0·5$ M $Na_4Fe(CN)_6$. The optimum quantity of electricity for the electrolysis was found to be 2×10^{-4} coulombs. The brain was then fixed overnight in 90% alcohol, sectioned at 20 μ, and stained with eosin.

Precipitates of Prussian blue about 20 μ in diameter were found at the site of the electrode tip.

INTRODUCTION

DURING an attempt to record activity from the hind-brain of 15-cm tench (*Tinca tinca*, Osteichthyes) with small diameter capillary electrodes, the need became apparent for a marking technique to permit accurate localization of the site of recording. The brain-tissue in lower vertebrates is too soft for micro-electrodes to leave tracks (Woldrings and Dirken, 1951). Further, if it were possible to cause the micro-electrodes to leave tracks, it is unlikely that precise localization of the tip region would be possible; nor is it possible with capillary micro-electrodes to use the accepted techniques for marking metal electrodes: release of metallic ions (Scheibel and Scheibel, 1956), or lesions produced by burning.

The ideal marking technique would have to satisfy several requirements. It should be simple to operate and not liable to cause movement of the electrode relative to the brain. The mark made should be distinct and specific to the site of the electrode tip. It was thought that the electrolytic release of ferrocyanide ions from the electrode and their precipitation as Prussian blue by a solution of ferric ions bathing the brain would meet these requirements. Subsequent histological investigation would permit accurate localization of the Prussian blue mark.

If the electrolyte in the micro-electrode contained ferrocyanide ions, the only procedure necessary would be to exchange the recording leads for leads from a convenient d.c. supply, and to add a ferric salt to the Ringer bathing the preparation. Neither of these operations would be liable to disturb the site

of the electrode tip and the mark produced would be permanent, distinct, specific, and readily identifiable.

<div align="center">PROCEDURE</div>

The micro-electrodes, made with a two-phase, vertical-pull machine (Winsbury, 1956) from 3-mm soft glass tubing, were filled with an electrolyte of the following composition: $2 \cdot 5$ M KCl$+$o$\cdot$5 M $Na_4Fe(CN)_6$ (Tasaki, Polley, and Orrego (1954)). Electrodes with an impedance of less than 3 or more than 35 megohms were rejected.

At the end of recording the electrode was left in position and the Ringer bathing the preparation was replaced by a saline solution containing ferric salts and left for not less than 4 h. The most satisfactory solution was found to be a mixture of equal parts of 110 mM ferric chloride and Ringer. This was made up immediately before use from a $1 \cdot 1$ M solution of ferric chloride, as in dilute solution ferric chloride rapidly hydrolyses to colloidal ferric hydroxide, which diffuses very slowly. The preparation was allowed to stand in this solution for not less than 4 h. When this was inconvenient it was found possible to reduce loss of histological detail by transferring the preparation and electrode, clamped together, to a refrigerator until it was convenient to carry out the rest of the marking procedure.

A minimum of 4 h was found necessary in order that the ferric ions could diffuse throughout the brain (distances up to 4 mm in this case). It was sometimes found that the slight fixing action of ferric chloride was sufficient to leave a conspicuous electrode track, but this was insufficiently reliable to be used by itself for site recognition. This is because tracks were not frequently found and, if present, could rarely be followed to the electrode tip; in some preparations they were readily confused with other artifacts produced in the brain by subsequent histological treatment.

At the end of the diffusion period, ferrocyanide ions were released electrolytically from the electrode. This was done by applying a potential difference of 4-V from a battery between inside (cathode) and outside (anode) of the micro-electrode. The optimum quantity of electricity was found to be 2×10^{-4} coulombs, i.e. with a 30 megohm micro-electrode the 4-V potential difference was applied for 30 min (current $=$ o$\cdot$13 μA for 1,800 sec) and with a 10 megohm micro-electrode this quantity was supplied in 10 min (o$\cdot$4 μA for 600 sec). The minimum quantity of electricity necessary was about 1×10^{-4} coulombs, but the use of twice this amount was found to give a more readily identifiable mark with greater consistency. The mark produced was not large enough to impair the required degree of accuracy and the use of 2×10^{-4}

FIG. 1 (plate). Photomicrographs of 20-μ transverse sections of tench hind-brain stained with eosin, showing the Prussian blue marks produced at the tip of micro-electrodes.

A, mid-facial lobe. There is a slight electrode tract and fixation artifact. 3×10^{-4} coulombs were used in electrolysis.

B, entry of spinal V-root. The mark at the tip of the electrode is clear but there has been some diffusion of ferrocyanide away from this region. 2×10^{-4} coulombs were used in electrolysis.

A

electrode track

site of electrode tip

fixation artifact

facial lobe

sensory VII fibres

decussating VIII fibres

fasciculus longitudinalis medialis

0·2 mm.

B

decussating VIII fibres

lemniscus lateralis

4th ventricle

spinal V root

site of electrode tip

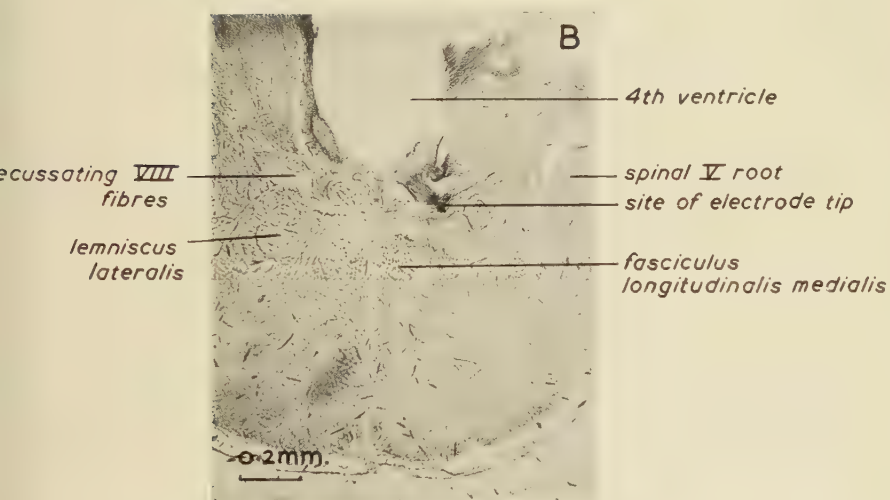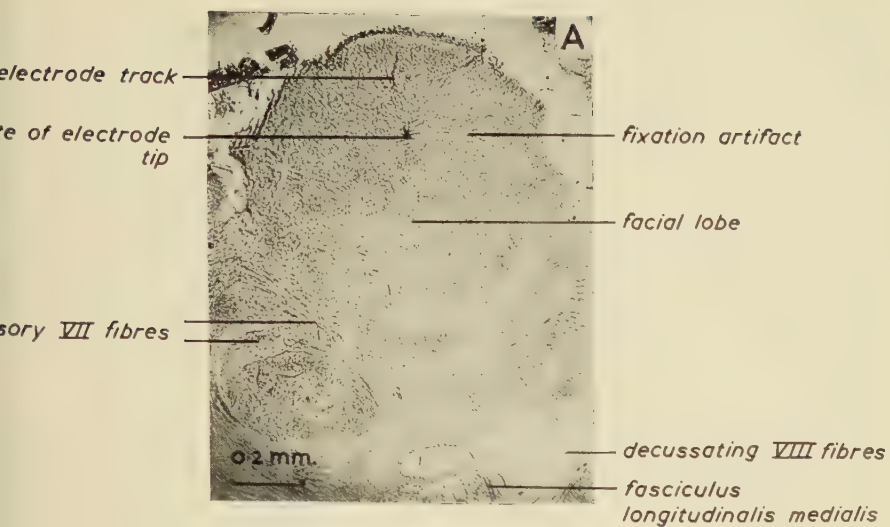fasciculus longitudinalis medialis

0·2mm.

Fig. 1

K. H. BULTIDUDE

coulombs also reduced the possibility of failure due to an undetected partial blocking of the electrode.

To prevent the diffusion of ferrocyanide ions away from the electrode tip it was found necessary to wait for complete diffusion of the ferric ions through the brain before starting the electrolysis. If this precaution was not observed, the marks were absent or a diffuse precipitate was formed.

The brain was fixed in 90% alcohol immediately after electrolysis. The optimum time for fixation was found to be about 15 h, i.e. overnight. The brains were then blocked in 58° C paraffin wax and serial transverse sections were cut at 20 μ through the relevant region. These were then stained lightly with eosin and mounted in DePeX.

RESULTS

Use of this technique has consistently produced a mark at the site of the electrode tip and the sections were found to show sufficient histological detail for the accurate identification of this site (fig. 1). The nervous elements stain in different intensities of red against which the precipitates of Prussian blue, 10–20 μ in diameter, produced at the electrode tip show clearly.

The technique is simple and not very time-consuming to operate; it is possible to observe the site of recording 30 h after recording has been made. The technique shares the disadvantage with other marking techniques that only the last site of recording can be marked for identification unless it is possible to use several electrodes on each preparation.

REFERENCES

SCHEIBEL, M., and SCHEIBEL, A., 1956. Stain Techn., **31**, 1.
TASAKI, I., POLLEY, H., and ORREGO, F., 1954. J. Neurophysiol., **17**, 454.
WINSBURY, G., 1956. Rev. Sci. Instr., **27**, 514.
WOLDRINGS, S., and DIRKEN, M., 1951. J. exp. Biol., **28**, 218.

A Bleaching Method for Melanin and two Staining Methods

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Summary

An acidified permanganate method can be used for bleaching melanin in mammalian skin and eyes. It allows good staining to be performed later.

Microfilariae in nitrocellulose sections can be stained by a modified phloxine tartrazine method.

Elastic fibres can be selectively stained by using a diluted solution of synthetic orcein.

A Method for bleaching Melanin

THE Mallory type of bleaching method involves the use of a solution of potassium permanganate followed by a solution of oxalic acid. Gomori (1941) used an acidified solution of potassium permanganate followed by a solution of sodium bisulphite as a preliminary to the staining with chrome alum haematoxylin and phloxin. We have found that this method, in a slightly modified form, is useful for bleaching melanin in paraffin and nitrocellulose sections.

(1) Transfer the sections from water to a freshly prepared mixture of equal parts of the following:

(*a*) 0·3% potassium permanganate.

(*b*) 0·3% sulphuric acid.

Leave the sections in this mixture until the melanin is bleached. This usually occurs in the following times:

skin	2 min
choroid and ciliary processes of the eye	15 min
iris of the eye	30 min

(2) Treat with 1% oxalic acid until the brown colour disappears.

(3) Rinse in two changes of distilled water.

(4) Wash in running tap water for at least 45 min.

(5) Rinse in distilled water.

(6) Stain as desired. Some staining times may be slightly altered.

This method is the only one that we know, which will bleach the melanin of the eye without seriously affecting subsequent staining methods.

A Method for staining Microfilariae in Nitrocellulose Sections

Lendrum (1947) introduced a phloxine tartrazine method which has been used by Jamison (Kershaw, Jamison, Nugent, and Duke, 1956) to show microfilariae in paraffin sections. This method in a modified form can be used to show them in nitrocellulose sections.

(1) Stain the sections with Weigert's iron haematoxylin for 3 min.

(2) Differentiate in 1% hydrochloric acid in 70% alcohol.

(3) Wash in several changes of tap water.

(4) Stain for 30 min in the following solution:

    phloxine   0·5 g

    calcium chloride 0·5 g

    distilled water   1000 ml

(5) Wash in distilled water.

(6) Transfer to the following solution for about 1 min or until most of the red colour has disappeared:

    tartrazine   0·5 g

    alcohol, 90%   100 ml

(7) Remove excess tartrazine with 90% alcohol.

(8) Clear and mount.

This method reveals microfilariae that would otherwise be difficult to notice. It works well after initial bleaching of the melanin with acidified permanganate solution. We have recommended this method to Dr. F. C. Rodger, who has used it in his studies of the eye in cases of onchocerciasis. We are indebted to him for the provision of the material.

A Method for staining Elastic Fibres

Prepare a stock solution of orcein by dissolving 0·5 g of orcein (synthetic, G. T. Gurr) in 100 ml of acid alcohol (70% alcohol containing 1% of hydrochloric acid). Leave overnight and then filter.

Treat paraffin sections as follows:

(1) Rinse in 70% alcohol.

(2) Stain overnight in a weak solution of orcein made by diluting 1 volume of the filtered stock solution with 10 to 20 volumes of acid alcohol.

(3) Rinse in acid alcohol.

(4) Wash in running tap water for 15 min.

(5) Counterstain with Weigert's iron haematoxylin if desired.

(6) Dehydrate, clear, and mount.

More intense staining of the elastic fibres may be obtained by using the acidified permanganate bleach before staining with orcein.

It is essential to use diluted stock solution and not to make up a dilute solution from the solid dye.

With this method, particularly after bleaching, even the finest sub-epidermal elastic fibres of the skin are stained a deep purple to black. Little or no background staining occurs after most fixatives.

REFERENCES

Gomori, G., 1941. Amer. J. Path., **17**, 395.

Kershaw, W. E., Jamison, D. G., Nugent, D., and Duke, B.O.L., 1956. Trans. Roy. Soc. trop. Med. Hyg., **50**, 6.

Lendrum, A. C., 1947. J. Path. Bact., **59**, 399.

A Simultaneous Coupling Azo Dye Technique Suitable for Whole Mounts

By P. R. LEWIS

(*From the Anatomy School, Cambridge*)

With one plate (fig. 1)

SUMMARY

A method based on the familiar coupling azo dye technique has been devised for the study of enzyme distribution in thick specimens. Its application to the study of esterase distribution in whole tadpoles of *Xenopus laevis* is described in detail. It can also be used to show the distribution of alkaline phosphatase, both in *Xenopus* tadpoles and in limbs dissected from chick embryos. It has proved useful in embryological studies both for teaching and for research.

ORDINARY section techniques have certain disadvantages when a three-dimensional picture is the primary requirement. These disadvantages are particularly serious in many studies of enzyme distribution, where ordinary paraffin embedding procedures cannot be used. An attempt was therefore made to design a suitable histochemical technique which would eliminate the need to cut sections.

This technique, which is described below, was originally devised to study the distribution of cholinesterase in developing tadpoles of the toad, *Xenopus laevis*. It has since been applied to other enzyme studies, and the general principle of it could be extended much farther.

The method is based upon the now widely used simultaneous coupling azo dye technique for esterases and phosphatases first introduced by Menten, Junge, and Green in 1944 and since improved and extended by Nachlas and Seligman (1949), Gomori (1952), Pearse (1954), and others. Thus, in the established technique for esterases, sections are incubated with α-naphthyl acetate in the presence of a suitable diazonium salt. As the esterase hydrolyses this substrate, free α-naphthol is released which very rapidly couples with the diazonium salt to give a brightly coloured precipitate of an azo dye at or close to the site of enzyme activity. The important modification made in applying this technique to whole tadpoles was to pre-incubate in a buffered solution of the diazonium salt before introducing the substrate, in order to ensure that an adequate concentration of the coupling agent was present throughout the tissue before any release of α-naphthol took place. Various minor modifications were also made in order to reduce the background staining of tissues by the diazonium salt, since even slight general coloration in a thick specimen seriously impairs the quality of the microscopic image. Thus the use of acetone to dissolve the substrate was eliminated, and phosphate

was replaced by tris(hydroxymethyl)aminomethane as the buffer. Sites of alkaline phosphatase activity can be demonstrated by a precisely similar method but with α-naphthyl phosphate in place of the acetate.

Details of Technique

The technique, as used to study cholinesterase distribution in the *Xenopus* tadpole, is as follows:

1. Fixation for 2–24 h in neutral 10% formalin at 4° C has proved most satisfactory.

2. After washing for a few minutes in tap-water, the tadpoles are skinned under a dissecting microscope. As much as possible of the intestine must also be removed, since this contains a very active esterase which is not rendered insoluble by the fixation employed.

3. After a total period of $\frac{1}{2}$ h in several changes of tap-water the tadpoles can be transferred to the diazonium salt solution for a period of 30–40 min. They are then transferred to the complete incubation medium and studied under a dissecting microscope. When the azo dye has reached the required intensity (usually after 2–20 min) they are returned to the diazonuim salt solution for 20–30 min. It should be noted that further intensification of the colour continues for several minutes after removal from the complete incubation medium.

4. After a thorough wash in water it is an advantage to fix the preparations in formaldehyde-calcium for at least a few days at room temperature before attempting to make permanent mounts.

5. Direct mounting of the preparations in glycerol produces gross distortion, because water diffuses out much more rapidly than glycerol diffuses in. This effect can be avoided by passing the tadpoles through a graded series of water-alcohol-glycerol mixtures. Alcohol reduces the viscosity of the water-glycerol mixtures and markedly reduces the rapid extraction of water from the tissues by the glycerol. The optimum number of mixtures in the series and the time taken in each will depend upon the size of the specimen. A typical series used is:

water:alcohol:glycerol by volume: $5:5:1 \rightarrow 5:5:3 \rightarrow 3:7:5$

$$\rightarrow 3:7:10 \rightarrow \tfrac{1}{2}:9\tfrac{1}{2}:10 \rightarrow \tfrac{1}{2}:9\tfrac{1}{2}:20.$$

This actual series was chosen merely because it was easily made up by mixing pure glycerol with the aqueous alcohol solutions kept in stock (50%, 70%, and 95%). The best results are obtained by placing tadpoles immersed in the final solution (preferably on cavity slides) in a desiccator over anhydrous calcium chloride, which rapidly takes up both water and ethyl alcohol.

6. Dissection of total preparations is not difficult, especially if the final dissecting is done in a glycerol-water mixture. Small pieces of tissue, obtained by blunt dissection, can be placed directly in the fourth solution of the series

given above on a microscope slide for final dissecting or teasing. Excess fluid can then be removed and the tissue mounted in glycerin jelly. Alternatively, the whole tadpole (or parts of it) can be embedded in gelatin and cut on the freezing microtome.

7. Supplementary techniques of various kinds can be applied before mounting. The azo dye is soluble in most lipid solvents, but not in water or ethyl alcohol at room temperature. Both the Bodian and Holmes silver techniques for nerve-fibres have been successfully applied to small pieces of tissue dissected from esterase preparations of tadpoles.

8. The solutions required are:

(a) The stock buffer solution, consisting of 0·2 M tris(hydroxymethyl)-aminomethane (M = 121; obtainable sufficiently pure from L. Light & Co., Colnbrook, Bucks.) made up in distilled water. This solution keeps at room temperature for several weeks or at 4° C for several months.

(b) A working buffer solution, best made up weekly:

> 10 ml stock buffer solution
> 1 ml N/1 hydrochloric acid   } pH = 8·1.
> 9 ml distilled water

(c) The diazonium salt solution:

> 3 ml buffer solution of pH = 8·1.
> 20–30 mg 'Red TR'.
> Made up to 10 ml, filtered, and used within a few hours.

The full designation of 'Red TR' is Brentamine fast red TR salt. It is the stable diazonium salt of 5-chloro-*o*-toluidine and can be obtained from the Academic Relations Department, Imperial Chemical Industries, Hexagon House, Blackley, Manchester, 9. Of the many diazonium salts tested, this one gave by far the best total preparations.

(d) The complete incubation medium, which should be made up immediately before use by the following procedure.

Place a few crystals (5–10 mg) of purified α-naphthyl acetate (British Drug Houses) in a test tube and pulverize them. (This is easily done by using a second, longer, and thinner test tube.) Add equal volumes (5–10 ml) of distilled water and unfiltered diazonium salt solution (c); cork the test tube, shake vigorously for ½ min, and filter. Some commercial samples of α-naphthyl acetate may need to be recrystallized from alcohol, but most samples can be adequately purified by two or three washes with very small volumes of alcohol.

For the study of alkaline phosphatase activity the same technique can be used but with sodium α-naphthyl phosphate (G. T. Gurr) as the substrate and sodium diethyl barbiturate as the buffer. Thus in place of solutions (c) and (d) above the following two solutions are used:

(e) 4 ml N/10 sodium diethyl barbiturate.

> 20–30 mg Red TR.
> Made up to 10 ml and filtered.

(f) 4 ml N/10 sodium diethyl barbiturate.
   10–15 mg Red TR.
   10 mg sodium α-naphthyl phosphate.
   Made up to 10 ml and filtered.

This procedure has also been used to demonstrate the alkaline phosphatase activity associated with calcification in bones dissected from the limbs of chick embryos, the dissection being made before fixation in cold formalin.

DISCUSSION

The successful application of the technique depends upon a number of conditions being fulfilled:

(*a*) Enzymes giving a positive reaction must not be destroyed by fixation and yet must be rendered insoluble in aqueous solutions. Structures found to contain any enzyme soluble after fixation must be removed before incubation, e.g. the intestine of the *Xenopus* tadpoles.

(*b*) The tissue must have an open structure to allow ready access of the reagents (otherwise activity in the superficial layers only will be revealed) and there must be no membranes present which are impermeable to either the substrate or the diazonium salt. These requirements can often be met by preliminary dissection before incubation, e.g. skinning of the tadpoles and dissection of the bones from chick limbs.

(*c*) The tissue must not contain a high proportion of fat, because the azo dye has an appreciable lipid solubility and fat tends to slow down diffusion of the reagents.

(*d*) The enzymes giving a positive reaction must be fairly sharply localized. If there is a significant amount of enzyme diffusely distributed it becomes difficult to distinguish any details in a thick specimen.

Just how far these conditions are fulfilled is usually obvious from a study of the specimen both during incubation and after final mounting in glycerol. As a further check, a treated specimen can be embedded in gelatin and frozen sections cut: it is then easy to assess how far adequate penetration of the reagents has occurred.

FIG. 1 (plate). A, B, and C are whole mounts, showing alkaline phosphatase distribution, prepared by students in an anatomy class. A, tibia and fibula; B, radius, ulna, and carpus, both from 8-day chick embryos; C, a culture of a phalanx.

D, part of a skinned, third-form *Xenopus* tadpole showing the esterase concentrated at the myocommata, where the myoneural junctions are. (The black area at the lower left is due to the melanin of the abdominal wall.)

E, the ciliary ganglion dissected from an esterase preparation of a *Xenopus* tadpole. The efferent nerve-fibres, which by-pass the ganglion and are presumably cholinergic, are much more deeply stained than the sensory neurones.

F, the end of a single myotomal muscle-fibre which had been teased out from an esterase preparation and silvered by the Bodian technique. The end of the muscle-fibre is deeply stained owing to esterase activity associated with the endings of the motor nerve; this is shown up by the silver stain.
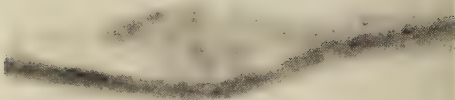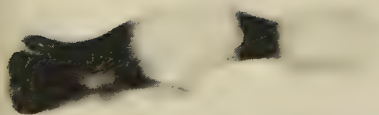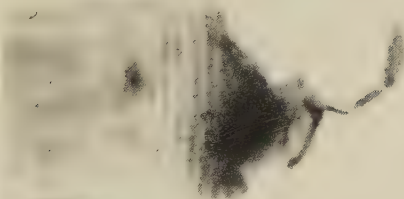
A 　　　　1mm

D 　　　　2 mm

1mm

E 　　　　50 μ

1mm

F 　　　　10μ

Fig. 1

R.P LEWIS

It should be emphasized that this technique was not designed for cytological studies of enzyme distribution, although the localization of the azo dye is often extremely good, particularly in superficial structures. Its chief value is as a general method, especially for following gross changes in the three-dimensional distribution of an enzyme. As such it has proved very useful in preliminary studies of enzyme changes during embryonic development (Lewis and Hughes, 1957). It has also proved useful for teaching several embryological problems to advanced students, e.g. the spread of alkaline phosphatase activity during calcification and progressive development of motor endings in skeletal musculature, in chick limbs and *Xenopus* tadpoles respectively. Examples from both research and teaching material are shown in fig. 1.

The complete incubation medium developed for the esterase technique (solution *d* described above) has also proved very suitable for studies in ordinary sections. It appears to be more stable than the one recommended by Pearse (1953) and to give less background staining due to decomposition of the diazonium salt.

REFERENCES

GOMORI, G., 1952. *Microscopic histochemistry.* Chicago (University Press).
LEWIS, P. R., and HUGHES, A. F. W., 1957. In *Metabolism of the nervous system.* London (Pergamon Press).
MENTEN, M. L., JUNGE, J., and GREEN, M. H., 1944. J. biol. Chem., **153**, 471.
NACHLAS, M. M., and SELIGMAN, A. M., 1949. J. Nat. Cancer Inst., **9**, 415.
PEARSE, A. G. E., 1953. *Histochemistry, theoretical and applied.* London (Churchill).
—— 1954. Internat. Rev. Cytol., **3**, 329.

Activity of the Brain/Corpora Cardiaca System during Pupal Diapause 'Break' in *Mimas tiliae* (Lepidoptera)

By K. C. HIGHNAM

(From the Department of Zoology, University of Sheffield)

With one plate (fig. 3)

SUMMARY

Pupal diapause in *Mimas tiliae* can be terminated by keeping the pupa for at least 4 weeks at 3° C. The adult emerges about 15 days after transfer to 25° C.

Histological examination shows that the neurosecretory cells in the brain are inactive in the diapausing pupa, but they elaborate intracellular material during the first 3 weeks at 3° C. The material is passed to the corpora cardiaca. The neurosecretory cells are again inactive by the end of the low-temperature period.

The brain/cardiaca system shows little sign of secretory activity during the subsequent period at 25° C. The corpora cardiaca undergo phagocytosis and reorganization during this time. This suggests that conditions for further development are established by the end of the low-temperature period. This hypothesis is supported by the fact that development of the non-endocrine organs begins immediately the pupa is transferred to 25° C after 4 weeks at 3° C.

Extirpation and implantation experiments involving the brain, with and without its associated corpora cardiaca, support the histological results, indicating that the brain is necessary for diapause development at 3° C and that the corpora cardiaca are involved in the release of the brain factor.

INTRODUCTION

THERE is evidence that diapause in insects is under the control of hormonal factors. *Rhodnius prolixus* nymphs live in a quiescent state (diapause) after decapitation, and it has been shown that the operation removes the source of developmental hormones (Wigglesworth, 1934). This author suggests that the diapause obtaining normally in many insect species results from the absence of developmental hormones. Further, Williams (1946) has shown that the termination of diapause in *Platysamia cecropia* is caused by factors secreted by the brain—factors produced mostly at low temperatures, but absent in diapausing pupae.

In *Bombyx mori* diapause in the egg is initiated by the sub-oesophageal ganglion of the parent (Fukuda, 1951*a, b*; Hasegawa, 1951). Possibly pupal diapause also is caused by the secretion of a diapause factor (Hinton, 1953).

It would appear then that in some cases diapause may result from the absence of a hormone; in other cases from the presence of one. Correspondingly, termination of diapause may result from the production of a hormone or the disappearance of one. Alternatively, the two processes of induction and termination of diapause may be due to the functioning of two different hormones.

The present investigation was begun in an attempt to resolve some aspects of this problem. It soon became evident that the process of diapause 'break' in *Mimas tiliae* differed in detail from that described by Williams (1946) in the *Platysamia cecropia* pupa. The description of this difference is the main concern of this paper. The histological observations are of particular interest since Williams has published no comparable data on the endocrine system in the pupa of *P. cecropia* during diapause and diapause 'break'.

<div align="center">MATERIAL AND METHODS</div>

Diapausing and experimentally produced post-diapause pupae of the Lime Hawk Moth, *M. tiliae*, were used.

Samples of the pupae for histological study were fixed in Bouin, Duboscq-Brazil, Susa, or Zenker. After fixation, the thick cuticle was dissected away from the tissues beneath, which were subsequently embedded in paraffin wax and sectioned at 6μ. A few pupae were embedded whole in ester wax after the cuticle had been softened with diaphanol. The serial sections were stained with Weigert's haematoxylin and eosin, with Masson's or Mallory's polychrome staining techniques, or with the chrome-haematoxylin phloxine technique of Gomori to show neurosecretory material. The haematoxylin and eosin method after Bouin fixation gave excellent results for the purpose of this study.

To give support to the results obtained from the histological study, a series of experiments involving removal or implantation of the brain and corpora cardiaca was performed. The methods followed will be described in the appropriate section (p. 82).

<div align="center">RESULTS</div>

Low temperature and the termination of diapause

Under natural conditions, the Lime Hawk Moth pupates during September and enters a pupal diapause lasting about 8 months, adult development beginning again about the middle of the following May. In the laboratory diapause can be extended to 12 months or longer by keeping the pupa in a saturated atmosphere at a temperature of $25°$ C. Although the period of diapause can be considerably lengthened by keeping the pupa at this temperature, it does not continue indefinitely. The adults always eventually emerge.

The diapause can be terminated precociously by subjecting the pupa to a low temperature ($3°$ C) for at least 4 weeks, and then transferring it to a temperature of $25°$ C; the adult emerges about a fortnight later (table 1). A number of pupae (10% in table 1) emerge after less than 4 weeks at $3°$ C, but the great majority require the full period. The process by which diapause is brought to an end is called 'diapause development' (Andrewartha, 1952). In the pupa of *M. tiliae* the rate of diapause development is evidently very low at $25°$ C, but is greatly increased at $3°$ C.

TABLE I

Adult emergence from pupae subjected to a temperature of 3° C

No. of pupae	Weeks at 3° C	No. of adults emerging after transference to 25° C	Days at 25° C before emergence
10	1	1	15
10	2	1	15
10	3	4	13–16
10	4	10	12–17
10	5	10	14–17
10	6	10	13–18

The figures in the last column represent days on which the first and last emergences in the samples occurred. In each sample, the largest number of emergences occurred during the fourteenth and fifteenth days.
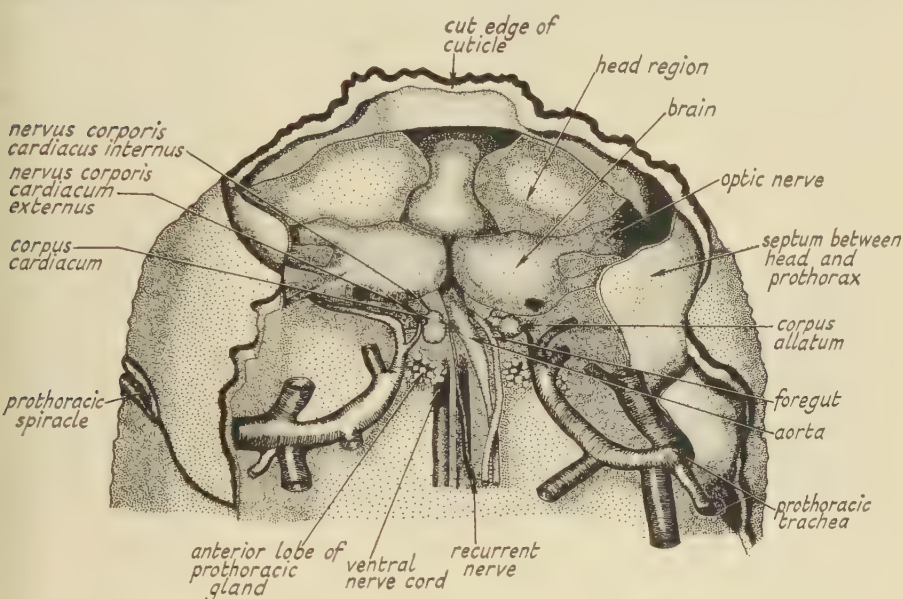

FIG. 1. Drawing of a dissection of the anterior part of the diapausing *M. tiliae* pupa to show the general arrangement of the endocrine system. Only the anterior lobes of the prothoracic glands are shown.

The histology of the neurosecretory cells and corpora cardiaca during diapause

The endocrine system has four components—neurosecretory cells (located in the cerebral ganglia), corpora cardiaca, corpora allata, and prothoracic glands. The *neurosecretory cells* are arranged in four groups, an inner and an outer group in each half of the brain. The inner groups lie close together on each side of the midline, on the dorsal side of the anterior region of the pars intercerebralis. Together they comprise 20 cells. Each outer group contains 5 cells and is situated dorsally about half-way along each cerebral lobe. The *corpora cardiaca* and *allata* are both paired (fig. 1), lying lateral to the aorta, from which

they are completely separate ('type latéralisé' of Cazal, 1948). Each corpus cardiacum is a small pyriform body, connected with the brain by the usual pair of nervi corporis cardiaci. The roughly spherical corpora allata lie immediately behind the corpora cardiaca so that no nervi allati are visible. The *prothoracic glands* consist of two bilaterally placed masses of tissue in intimate association with the branches of the prothoracic tracheae. In general appearance they resemble a tightly clumped string of beads. Fig. 1 shows the appearance of part of the tissue lying posterior to the brain.

The neurosecretory cells. The cells are generally pear-shaped in outline with a prominent axon leaving the cell at the apex of the narrow end. They are readily identified by their staining reactions, the cytoplasm becoming deep pink or red with eosin, red with acid fuchsin, and reddish blue with chrome haematoxylin. There are, however, two distinct types of cell, different in size and with some difference in staining reaction. The first type is small (mean diameter about 24 μ) with a markedly acidophil cytoplasm and an extranuclear meshwork which stains strongly with haematoxylin. The second type is larger (figs. 2, A; 3, A), with a homogeneous acidophil cytoplasm containing a few acidophil globules or clear vacuoles. The extranuclear meshwork is less obvious than in the smaller cells. There are more of the smaller type of cell, but both types are present in the several groups of neurosecretory tissue. No sign of any secretory material can be found along the paths of the nervi cardiaci at this time. The neurosecretory cells (fig. 3, B) in the brains of a small number of diapausing individuals show signs of the sort of secretory activity which will presently be described. It is thought that these are the individuals which break diapause prematurely when subjected to a low temperature for periods of less than 4 weeks (p. 74 and table 1).

The corpora cardiaca. Each corpus cardiacum (fig. 6, A, p. 81) is surrounded by a thin membrane which stains blue or green with Mallory's or Masson's technique. The membrane is continuous with the thicker membrane over the corpus allatum. A large part of each gland is made up of the nerve-fibres from the nervi corporis cardiaci (fig. 6, A). Numbers of nerve-cells are also present, especially where the nervi cardiaci enter the gland. A few connective tissue-cells beneath the bounding membrane and cells of the tracheal epithelium can be identified.

In the posterior part of each corpus cardiacum are four or five large cells (fig. 6, A), very different in appearance from the other components of the gland.

FIG. 3 (plate). Photomicrographs of neurosecretory cells in the pars intercerebralis.

A, diapausing pupa. Two cells with obvious extranuclear meshworks are shown. No inclusions (stage *b*).

B, diapausing pupa. Cells with inclusions (present in a very small proportion of the diapausing pupae).

C, after 1 week at 3° C. Cell with small inclusions shown, together with *a* and *b* cells.

D, after 3 weeks at 3° C. *a*, *c*, and *d* cells shown.

E, after 4 weeks at 3° C. Only *a* cells present.

F, neurosecretory material along the axons of the nervus corporis cardiacum.

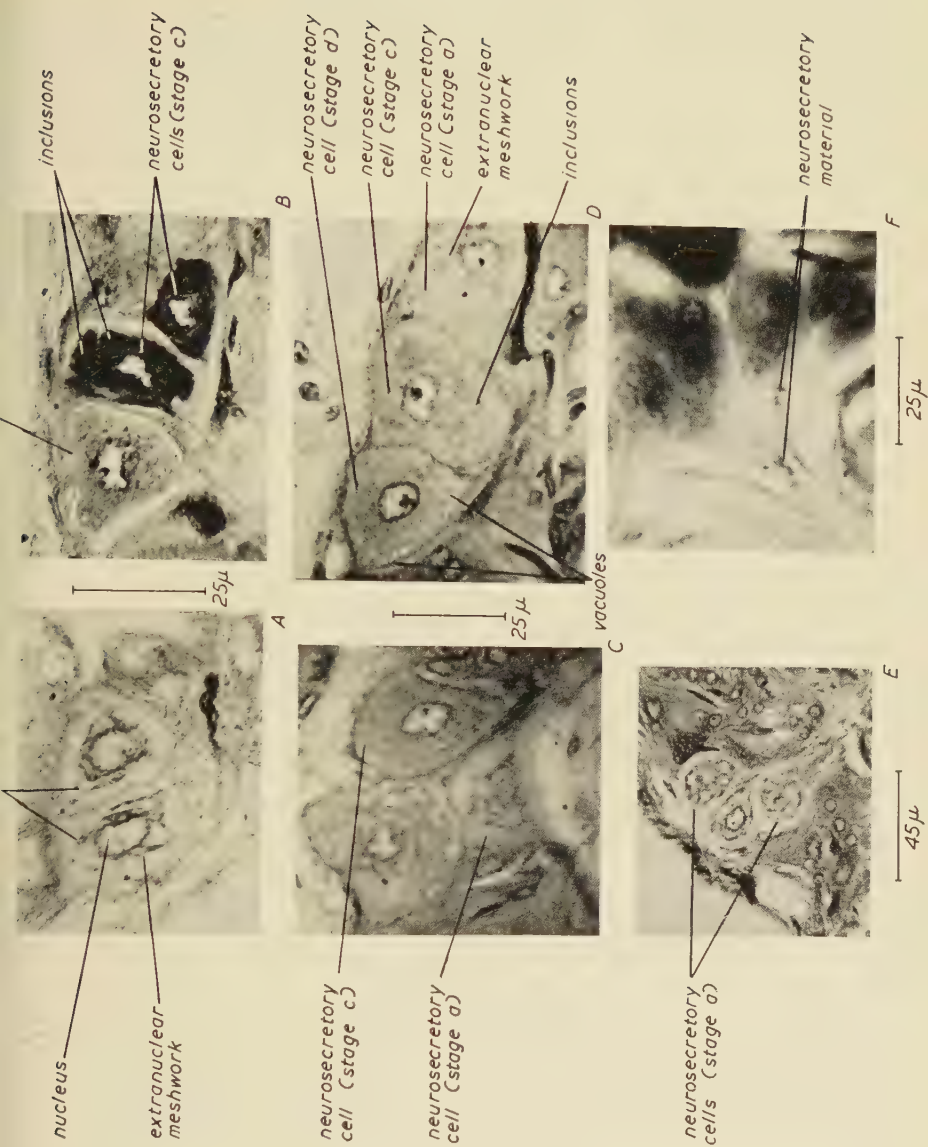A, B, and F, Masson's trichrome. C, D, and E, Weigert's haematoxylin and eosin.

FIG. 3

K. C. HIGHNAM

The cells have a mean diameter of about 35 μ, with one or two long prolonga-
tions ending blindly in the tissue of the gland. Around the nucleus and spread-
ing through the cytoplasm of each cell are traces of a meshwork similar to that
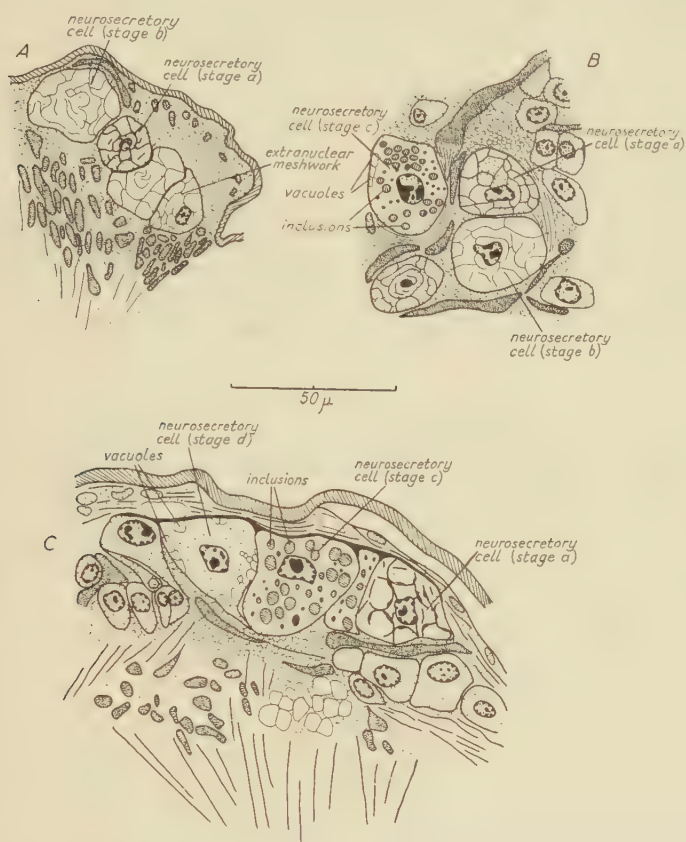described from the neurosecretory cells in the brain. These large cells are

FIG. 2. Camera lucida drawings of sections of brain through the pars inter-
cerebralis region to show the neurosecretory cells. A, vertical section,
diapausing pupa. The neurosecretory cells contain no inclusions and the
extranuclear meshwork is very obvious (stages *a* and *b* of the secretory
cycle). B, horizontal section, after 1 week at 3° C. One cell contains inclusions
and a few peripheral vacuoles (end of stage *c*). C, vertical section, after 2
weeks at 3° C. Three cells in different stages of the secretory cycle are shown.

thought to be the glandular elements of the corpora cardiaca. Since the cor-
pora cardiaca develop as evaginations from the foregut in the neighbourhood
of the hypocerebral ganglion (Roonwal, 1937), they are considered to be
modified autonomic nerve-ganglia. On this basis, the large cells in the corpora
cardiaca are possibly modified nerve-cells, and therefore analogous to the
neurosecretory cells in the central nervous system, which they resemble.

The histology of the neurosecretory cells and corpora cardiaca during diapause development at 3° C

The neurosecretory cells. By the end of the first week at 3° C, the staining reactions of the neurosecretory cells are very intense, enabling the cells to be easily identified. Close examination shows that the same two types of cell are present that were identifiable in the brain of the diapausing pupa (figs. 2, B; 3, C) but these now constitute a smaller proportion of the total number of cells (table 2). The greater number of the cells contain masses of inclusions, while others are vacuolated (fig. 2, B). These cells are not of new origin, since the total number of cells remains unchanged. These different neurosecretory cells are thought to represent stages in an asynchronous cycle of elaboration and release of the intracellular material. The cycle can be differentiated into 4 stages, according to the size of the cells (table 2) and their contents (fig. 4). Though it is not possible to correlate the changes in each cell with a definite time sequence, examination of the material suggests the cycle to be as follows.

TABLE 2

Size of neurosecretory cells at different stages in the secretory cycle, and the proportion of different stages present during diapause and diapause development at 3° C

			% present during diapause and diapause development			
Stage	*Mean diameter (μ)*	*Diapausing*	*After 1 week at 3° C*	*After 2 weeks at 3° C*	*After 3 weeks at 3° C*	*After 4 weeks at 3° C*
a	24	} 95	44	47	50	84
b	27					
c	32	} 5	56	53	50	16
d	27					

The 'resting' stage (stage *a*) in which each cell is small (figs. 2, B; 4, A) with a mean diameter of about 24μ (table 2). The nucleus is small and globular. The cytoplasm takes up stain rather poorly, when compared with later stages. Around the nucleus and extending through the cytoplasm is a meshwork of interconnected basiphil strands. This stage corresponds almost exactly with the smaller kind of neurosecretory cell described from the diapausing pupa.

The second stage (stage *b*) only differs from the preceding one in that it is slightly larger (table 2), and the cytoplasm stains more deeply. At the points of junction of the extranuclear meshwork are small globules of intensely basiphil material (fig. 4, B). This stage is similar to the second type of 'diapausing' cell.

The next stage (stage *c*) includes several intergrading phases which show a progressive accumulation of intracellular material. The basiphil globules which are present in stage *b* at the junctions of the meshwork strands are considerably larger, and smaller globules are apparent along the lengths of the

strands. Acidophil globules almost completely fill the cell in many cases (figs.
2, B; 4, C), and in these the meshwork has disappeared, or is perhaps hidden
by the accumulated intracellular material. At the end of this stage, the cell is
much larger (table 2). Its nucleus, too, is larger than in either of the preceding
stages, and may be distorted from a spherical shape, possibly as a result of the
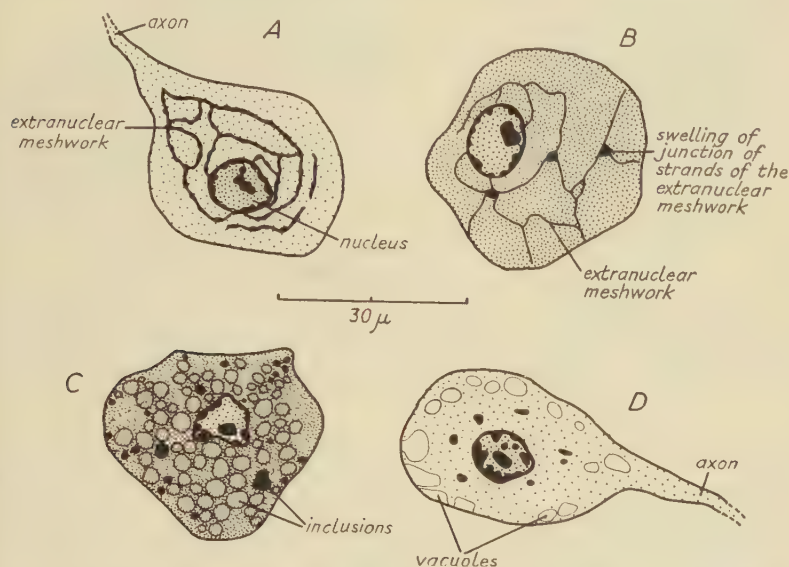pressure of the elaborated material in the cell (fig. 4, C).

Fɪɢ. 4. Camera lucida drawings of neurosecretory cells from the pars intercerebralis region to
show the 4 stages of the secretory cycle. Semi-diagrammatic.

No intracellular material is present in the fourth stage (stage *d*), and the
cytoplasm is vacuolated, especially around the cell periphery (fig. 4, D). Some
cells with few vacuoles have the proximal part of the axon swollen and packed
with intracellular material, which can also be seen along the lengths of the nervi
corporis cardiaci (fig. 3, F), whose origins lie in the neurosecretory cell groups.
There is little doubt that this stage is derived from the preceding one (stage *c*)
by the passage of elaborated material from the cell-body along the axons.
The cell has decreased from the size shown in stage *c* (table 2).

Stage *c* and *d* cells are present (figs. 2, C; 3, C, D; 5, A) in about the same
proportion after 2 and 3 weeks at 3° C (table 2). It is probable, therefore, that
there is no accumulation of material within the groups of neurosecretory cells
as a whole. It is possible that the rate at which material is produced may change
during the first 3 weeks at 3° C, but no evidence for such a change can be
obtained by the present method of analysis, which gives only the proportions
of cells at different stages present at the time of fixing. But no matter what the
rate of production is, the material is passed from the cell-bodies after forma-
tion, as can be seen by its presence along the nervi cardiaci.

By the end of the fourth week at 3° C, stage *c* and *d* cells are no longer visible

in quantity (table 2). The majority of the cells are in stage a, with a few in b...
The general appearance of the groups of cells is very similar to that in the
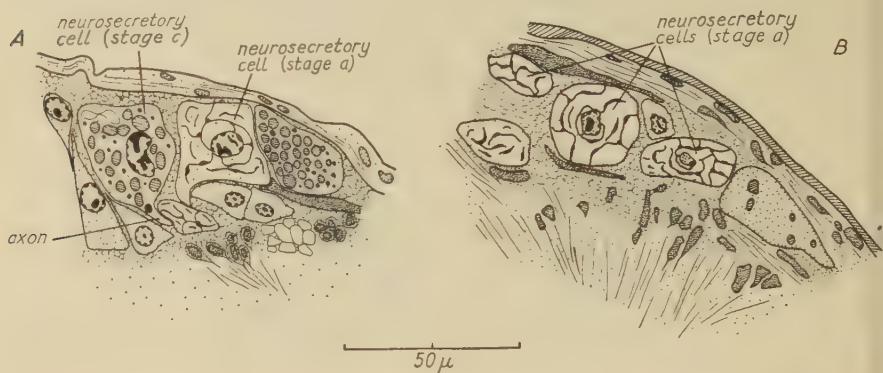diapausing pupa (figs. 3, E; 5, B; compare figs. 2, A; 3, A).

FIG. 5. Camera lucida drawings of brain sections through the pars intercerebralis region.
A, vertical section, after 3 weeks at 3° C. Neurosecretory cells in three stages of the secretory
cycle similar to fig. 2, C. B, vertical section, after 4 weeks at 3° C. One cell only shows a few
inclusions. Most cells are in stage a of the cycle (similar to fig. 2, A).

The appearance of the neurosecretory cells in the brain during diapause
and after 4 weeks at 3° C, compared with their appearance after 1, 2, and 3
weeks at this temperature, indicates that intracellular material either is not
being produced in the diapausing pupa and in the pupa after 4 weeks at 3° C.,
or is being produced at such a slow rate, or by so few cells, that stage c cells
are not obvious. Further, the transference of the diapausing pupa to the low
temperature in some way initiates or speeds up the production and release of
the material by the neurosecretory cells (so that stage c and d cells are visible
in quantity) during the first 3 weeks (table 2). Production of the material
finishes abruptly during the fourth week at the low temperature.

Scharrer (1941) and Dupont-Raabe (1951) described comparable stages
in the elaboration of intracellular material in the neurosecretory cells in the
brains of cockroaches and phasmids respectively. Both authors assume that
the smaller cells without inclusions or vacuoles represent a stage near the
beginning of the secretory cycle, before the accumulation of material has begun.

The corpora cardiaca. By the end of the first week at 3° C, the corpora
cardiaca appear rather larger than the glands in the diapausing pupa (fig. 6,
B). There is little difference between the glandular cells of the corpora cardiaca
at this time compared with those in the diapausing pupa. But a characteristic
feature of the glands now is the presence of large numbers of intensely acido-
phil globules, sometimes in vacuoles, in the body of each gland. The globules
are of various sizes, the largest being almost 5μ in diameter. They are espe-
cially abundant near the points of entry of the nervi corporis cardiaci (fig. 6, B).
Similar material can also be seen in association with the fibres of these nerves.
It would seem that the material elaborated by the neurosecretory cells in the

brain is being transferred along the nervi cardiaci to the corpora cardiaca. Such transfer has been shown to occur in other insect species (Scharrer, 1946, 1952a, b; Thomsen, 1954). Nayar (1954) describes cytoplasmic inclusions (spheroids) in the corpora cardiaca of adult *Locusta migratoria*, but it is not clear whether the spheroids are neurosecretory material from the brain stored in the corpora cardiaca, or whether they are a secretion product of the glands themselves.
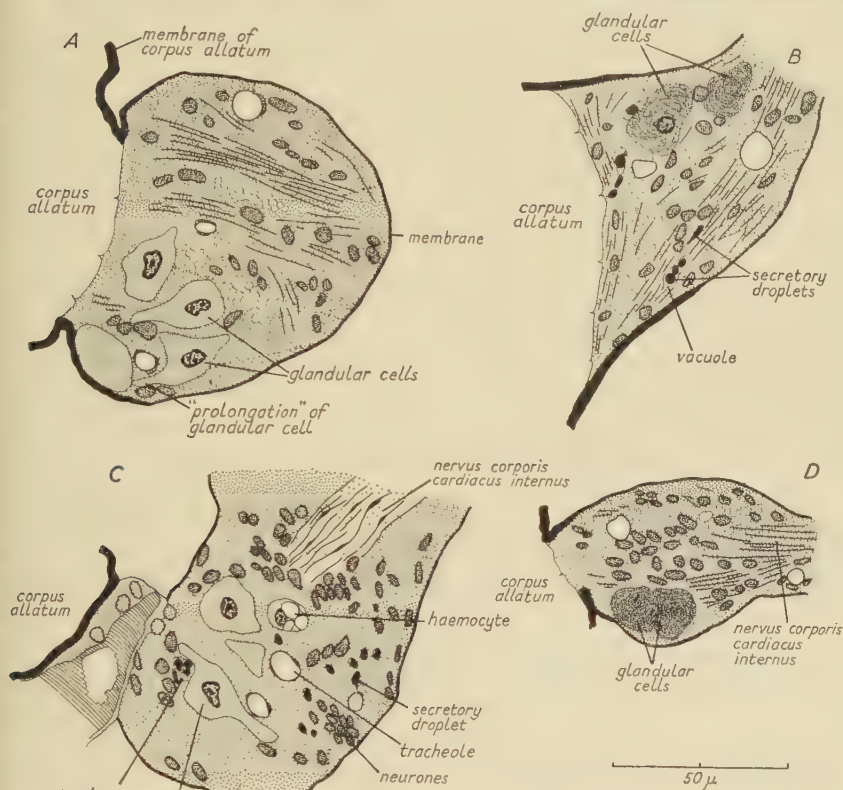

Fig. 6. Camera lucida drawings of sections of corpus cardiacum. A, in the diapausing pupa. The gland is large, but there are no inclusions. B, after 1 week at 3° C. Note the presence of acidophil inclusions, especially in the region of the nervus corporis cardiacus. There is a fine basiphil extranuclear meshwork in the cytoplasm of the glandular cells. C, after 3 weeks at 3° C. The gland is still large and the acidophil inclusions are numerous. A vacuolated haemocyte is evident near the centre of the gland. D, after 4 weeks at 3° C. The gland is small compared with the previous stage. There are no inclusions. The glandular cells are small and relatively inconspicuous.

The spheroids differ considerably in size and appearance from the masses of large acidophil inclusions which are present in the corpora cardiaca of nymphs of *Periplaneta americana* shortly before moulting (unpublished observation), which more nearly resemble the globules in the corpora cardiaca of the pupa of *M. tiliae* described here. The material in the corpora cardiaca in the pupa

of *M. tiliae* is present until the end of the third week at 3° C (fig. 6, c), but by the end of the fourth week the corpora cardiaca are very much smaller, the glandular cells are small and stain feebly, and there is no sign of any acidophil material in the glands (fig. 6, D). This agrees closely with the absence of the neurosecretory material in the brain at this time (p. 80). It is to be inferred from these observations that the material elaborated by the neurosecretory cells in the brain is passed to the corpora cardiaca, but does not remain long in the glands. It is difficult to demonstrate how the material leaves the glands. The membrane around the corpora cardiaca is incomplete in places, and it is possible that the material contained in the glands is passed directly out into the blood. In a few series (fig. 6, c), however, vacuolated haemocytes have been found inside the corpora cardiaca, and it is possible that the material within the glands is actively removed by these blood-cells (see Wigglesworth 1955a).

Beginning of post-diapause development in non-endocrine organs of the body

The hypodermis, thoracic muscles, and anterior imaginal ring (of the alimentary canal) were examined in detail throughout the diapause and diapause development periods. During these times the histological appearance of the tissues remains unchanged. The low temperature produces no visible effect upon them. But almost immediately the pupa is transferred to a temperature of 25° C (after at least 4 weeks at 3° C) intense mitotic activity begins in all three tissues, followed by differentiation and development until the adult form is attained. This immediate development of the non-endocrine organs of the body is due to the establishment of conditions favouring development by the end of the prior low-temperature treatment. Further evidence for this is given by the fact that at the subsequent high-temperature period no secretory activity of any part of the endocrine system is visible (except a short burst of activity by the neurosecretory cells in the brain at about the sixth day after transfer to the high temperature, an activity that can have nothing to do with the *initiation* of development in the general body-tissues). Indeed, during the period at 25° C before adult emergence, the corpora cardiaca and allata are invaded by phagocytic blood-cells, and undergo a process of reorganization very similar to that occurring in the non-endocrine organs. The prothoracic glands disappear entirely, after being invaded by blood-cells, by about the twelfth day after the transfer to the high temperature.

Removal and implantation of the brain and the corpora cardiaca

Several series of experiments were carried out on the extirpation and re-implantation of the various parts of the endocrine system in order to follow more closely its effect in terminating diapause.

The operations were carried out on pupae anaesthetized with ether. The pupae were placed in a block of wax which had been hollowed out at an angle to the vertical to form a pit of such form and size that each specimen was held

securely, with only the anterior part of the body projecting above the wax. On one side of this deep pit was a shallow depression which was kept filled with physiological saline.

A roughly rectangular area of cuticle was cut out from the dorsal side of the head, and this was placed in the saline in the shallow depression. The appropriate organs were then removed. Any loss of blood which had occurred was made good by the addition of physiological saline. A few crystals of phenyl-thiourea were placed in the body of the pupa to prevent the browning and eventual death of the operated animals, which results from the enhanced activity of the tyrosine-tyrosinase system after wounding (Williams, 1947). The cuticle was then replaced, the surface of the pupa dried, and the cut edges sealed with molten paraffin wax. Finally, the animals were placed on cotton-wool in an atmosphere saturated with water vapour.

Extirpation of the brain from the diapausing pupa. Two series of 10 diapausing pupae were operated upon for the removal of the brain. After removal of the cuticle from the dorsal part of the head, the brain with the corpora allata and cardiaca was immediately obvious. The nervi corporis cardiaci were severed, as also were the circum-oesophageal connectives. The nervous tissue making up the brain and sub-oesophageal ganglion is very condensed in the pupa of *M. tiliae* and it is consequently difficult to identify the circum-oesophageal connectives as such. In all cases the cuts were made at about the middle of the lateral nervous tissue. All other nerve connexions with the anterior parts of the body were cut and the brain lifted out. The cuticle was replaced and sealed into position, and the pupa placed on cotton-wool in a saturated atmosphere and stored in a refrigerator at 3° C for 4 weeks. The sample was then transferred to 25° C.

None of the pupae died during the low-temperature treatment, but 12 of the total of 20 died during the first week at 25° C. The remaining 8 survived for more than 2 weeks.

Ten control animals were subjected to the same operation except that the brains were not removed. Three of these control pupae died in about 2 weeks at 25° C.

Two of the brainless pupae were dissected at the end of the second week at 25° C. They were found to be almost identical in internal appearance (green blood, compact fat-body, no thoracic musculature, &c.) with normal diapausing pupae and showed no sign of adult development. Unoperated pupae, subjected to the same temperature treatments as these brainless pupae, would have been almost ready for hatching by this time (table 1). The remaining brainless pupae survived for a further 2 weeks at 25° C without any sign of adult development. Histological examination of the tissues confirmed this result.

The 7 surviving operated control pupae also had not hatched after 15 days at 25° C (the normal time for hatching after previously chilling for 4 weeks). But dissection revealed that adult development had proceeded normally, since the blood was creamy (being filled with haemocytes and fat-body cells),

and adult hypodermis (with scales) and thoracic musculature were well developed. Diapause had been broken in these pupae, although the normal hatching process had not proceeded to completion.

It may be concluded from this experiment that the brain is necessary for diapause development in the *tiliae* pupa, and that only when the brain is present can the low temperature cause diapause 'break'. These results agree with those of Williams (1946) on the *cecropia* pupae.

Implantion of chilled brains alone into diapausing pupae. Ten diapausing pupae, previously maintained at 25° C in a saturated atmosphere, were each implanted with a brain taken from a pupa which had been subjected to a temperature of 3° C for not less than 4 weeks. Care was taken during the removal of the 'chilled' brains to exclude all traces of the corpora cardiaca and allata. Implantation was carried out by anaesthetizing the experimental pupae and making a small slit in the arthrodial membrane between two abdominal terga. The 'chilled' brain was pushed through the slit into the body, a few crystals of phenylthiourea added, and the slit sealed with molten paraffin wax. The insects were then returned to the saturated atmosphere at 25° C. Nine of the 10 animals successfully survived the operation.

Fifteen days after the implantation none of the surviving pupae had hatched. Three were dissected and it was found that the chilled brain implant had 'taken' satisfactorily, tracheae having grown into the implant from the host. Otherwise the animals were identical with untreated diapausing individuals (green blood, compact fat-body, no thoracic musculature, &c.). No hatching occurred in the following two months. At the end of this period the survivors were dissected, but none showed any sign of adult development.

This result was quite unexpected, in view of the results obtained by Williams (1946) from similar experiments with the pupa of *Platysamia cecropia*. In this species Williams found that implanted chilled brains would terminate diapause in unchilled diapausing hosts. There would appear to be three possible explanations of the result of the present experiment.

(*a*) The implanted chilled brains had been unaffected by the low temperature to which they had been previously subjected. This would seem to be unlikely, in view of the results from the brain extirpation experiment (p. 83).

(*b*) The factor had been produced by the implanted brain during the low temperature, but could not be released into the blood of the diapausing host. Scharrer (1946, 1952*a*, *b*) has shown that section of the nervi corporis cardiaci in *Leucophaea maderae* acts like a ligature in preventing the exit of neurosecretory material. The nervi cardiaci were cut when chilled brains were removed from the *M. tiliae* pupae. It is possible, therefore, that failure of the brain factor to pass the cut ends of the nerves accounts for the results of the present experiment. This effect of section of the nervi cardiaci cannot be of universal occurrence, however, since unchilled *Platysamia cecropia* pupae will terminate diapause when implanted with chilled brains (Williams, 1946). This indicates that the factor is being released from the brain, although the nervi cardiaci have presumably been cut.

(*c*) The factor had been produced by the brain during the low-temperature period, but was no longer present at the time of the operation. This is the most likely possibility for the histological results already described (p. 80) indicate that the material produced by the neurosecretory cells in the brain is passed along the nervi cardiaci to the corpora cardiaca before the end of the low-temperature period. The observation suggested the following experiment.

Implantation of chilled brains, corpora cardiaca, and corpora allata into diapausing pupae. In this experiment it would have been desirable to implant only the brain and corpora cardiaca. Unfortunately this proved impossible owing to the intimate association between the corpora cardiaca and allata and the consequent difficulty of separating them completely. It is unlikely, however, that the inclusion of the corpora allata has influenced the result, since the small size of the glands and the absence of inclusions and vacuoles, &c., at the time of the operation indicates that they are not actively secreting (unpublished observation).

The brain with the attached corpora cardiaca and allata were removed from the pupae previously chilled for 24–28 days and implanted into the abdomens of pupae diapausing at 25° C. Fifteen implants were made into a similar number of diapausing pupae. Both host and donor pupae were then transferred to a saturated atmosphere at 25° C. The subsequent development of the host pupae is described below; that of the donor pupae in the next section.

Eleven host pupae survived for 4 weeks or longer at the high temperature, but none hatched out. When dissected, however, it was found that in 7 of the 11 diapause had been broken, whereas in the other 4 there was no sign of adult development. In the first group the blood was creamy, the thoracic leg- and wing-muscles were developing, and the hypodermis possessed scales. In the second group the blood was still clear and green and the fat-body compact.

It is thus clear that whilst the implantation of chilled brains alone into diapausing pupae will not break diapause, the implantation of chilled brains plus corpora cardiaca and allata may do so. The failure of this latter treatment to break diapause in four of the experimental pupae suggests that the factor responsible for this event was not available in these particular cases. The most probable explanation for this is that the factor had already been released into the blood of the donor and was thus not available to the host pupa. If so, the donor pupae might be expected to break diapause despite the loss of the brain-gland complex; evidence presented in the next section indicates that this did in fact occur. Thus 24–28 days appears to be the critical time for carrying out transplantation after exposure to 3° C.

Extirpation of the brain, corpora cardiaca, and corpora allata from chilled pupae. The animals from which the chilled brains and glands were removed in the previous experiment were subsequently kept at 25° C. Twelve of the fifteen pupae survived for more than 15 days after the operation. No adults emerged from any of the pupae, but dissection of the animals on death revealed interesting differences between two groups of pupae. Nine of the animals, although dehydrated, possessed a compact fat-body identical with that of the

diapausing pupa. The other three were dark-brown or black internally, and showed no sign of a well-organized fat-body. The cuticle was thin in the latter group and there were signs of developing thoracic musculature. Adult development had begun in 3 of the operated animals, whereas it had not in the other 9. This is the complement of the results of the previous experiment (p. 85). It indicates that the factor initiating development leaves the brain–gland complex soon after the 24th day at 3° C.

DISCUSSION

The marked difference between the appearance of the neurosecretory cells during the diapause and low-temperature periods is a very interesting feature of the cells in the pupa of *M. tiliae*, since such differences have rarely been described before. In *Rhodnius prolixus* Wigglesworth (1940) states '. . . there are no differences in the [neurosecretory] cells during fasting and at the height of secretion of the moulting hormone'; i.e. the appearance of the neurosecretory cells does not differ between their resting and active phases which have been experimentally determined. Similarly, Scharrer (1941) was unable to find any rhythmic activity of the groups of neurosecretory cells as a whole in the cockroach species which she examined. More recently, however, Dupont-Raabe (1951) has demonstrated in the phasmid *Cuniculina annamensis* that the elaboration of intracellular material by the neurosecretory cells in the brain is particularly intense a certain time after each larval moult, and also during egg-laying in the adult. Jones (1956) has shown that the neurosecretory cells in the embryo *Locustana pardalina* reach a period of maximal activity on the fourth day after the quiescent egg has been wetted, and Fraser (1957), working with *Lucilia caesar*, has shown differences in activity during diapause and after in several types of cerebral neurosecretory cell. The conclusions drawn from the examination of the neurosecretory cells in *tiliae* are in accord with these recent studies.

The present histological observations indicate that the production of material by the neurosecretory cells in the brain is not proceeding, or is proceeding very slowly, during the diapause period. Chilling the pupa stimulates the production of intracellular material, and this is then passed to the corpora cardiaca, where it is stored for some little time. The period of diapause development in the pupa of *M. tiliae* is thus correlated with the production of material by the neurosecretory cells in the brain. The intracellular material from these neurosecretory cells is related in some way to the hormone (or hormones) produced by the brain which influences growth and development throughout the life of the insect (Wigglesworth, 1955b). It seems that conventional techniques stain a biologically inert 'carrier-substance' (Clarke, 1956) and not the active brain factor itself; but it is reasonable to conclude that when the carrier-substance is produced, so is the associated hormone.

The conclusions arrived at from the examination of the neurosecretory cells and the corpora cardiaca during diapause development, together with

the results of implantation and removal of these organs, suggest that the conditions governing the further development of the pupa are established by the end of the low-temperature period. This hypothesis is supported by the fact that the non-endocrine organs of the body (e.g. hypodermis, thoracic muscles, anterior imaginal ring) begin development almost immediately the pupa is transferred to 25° C after at least 4 weeks at 3° C.

In *Platysamia cecropia* (Williams, 1946, 1947, 1948, 1949) the diapausing pupa has to be kept at the low temperature for a minimum period of 6 weeks, and diapause is then terminated after a further 2 weeks at 25° C. Williams postulated that during the 2 weeks at 25° C the actual release of the brain factor and stimulation of the prothoracic glands takes place. *M. tiliae* differs from *Platysamia cecropia* in this respect, since the present results show that no 'latent period' at 25° C is necessary in this animal before adult development begins. Recently Williams (1956) has shown that by greatly extending the low-temperature period, the time at 25° C before diapause is broken can be reduced to as little as one day. The difference between *M. tiliae* and *Platysamia cecropia* therefore seems to be that the brain factor is released slowly at low temperatures in *P. cecropia* and rapidly in *M. tiliae*. A consequence of the fact that the brain factor is released slowly at the low temperature in *Platysamia cecropia* is that the brain of a pupa chilled for 6 weeks will initiate development when implanted into an unchilled diapausing pupa (Williams, 1946). The present histological evidence suggests that no hormone is present, or is produced, in the brain of the *M. tiliae* pupa at the end of the low-temperature period and that therefore the brains of pupae chilled for 4 weeks cannot initiate development when implanted into diapausing pupae. The experimental implantation of chilled brains (p. 84) supports the histological evidence.

It is assumed that the effect of the brain factor in initiating development is mediated through the prothoracic glands. This has not yet been tested experimentally, but the experiments of Williams (1947) together with histological observations on the activity of the prothoracic glands (to be described in a later paper) support the assumption.

REFERENCES

ANDREWARTHA, H. G., 1952. Biol. Rev., **27**, 50.
CAZAL, P., 1948. Bull. biol., Suppl. **32**, 1.
CLARKE, R. B., 1956. Kongl. Fys. Sällsk. i Lund Forh., **26**, 1.
DUPONT-RAABE, M., 1951. Bull. Soc. zool. Fr., **56**, 386.
FRASER, A., 1957. Nature, **179**, 257.
FUKUDA, S., 1951a. Proc. imp. Acad. Japan, **27**, 582.
—— 1951b. Ibid., **27**, 672.
HASEGAWA, K., 1951. Ibid., **27**, 667.

HINTON, H. E., 1953. Entomologist, **86,** 279.

JONES, B. M., 1956. J. exp. Biol., **33,** 175.

NAYAR, K. K., 1954. Quart. J. micr. Sci., **95,** 245.

ROONWAL, M. L., 1937. Phil. Trans. B., **227,** 175.

SCHARRER, B., 1941. J. comp. Neurol., **74,** 93.

—— 1946. Anat. Rec., **96,** 577.

—— 1952a. Ibid., **112,** 386.

—— 1952b. Biol. Bull. Wood's Hole, **102,** 261.

THOMSEN, E., 1954. J. exp. Biol., **31,** 322.

WIGGLESWORTH, V. B., 1934. Quart. J. micr. Sci., **77,** 191.

—— 1940. J. exp. Biol., **17,** 201.

—— 1955a. Ibid., **32,** 649.

—— 1955b. *Physiology of insect metamorphosis.* Cambridge (University Press).

WILLIAMS, C. M., 1946. Biol. Bull. Wood's Hole, **90,** 234.

—— 1947. Ibid., **93,** 90.

—— 1948. Ibid., **94,** 60.

—— 1949. Bull. biol., Suppl. **33,** 52.

—— 1956. Biol. Bull. Wood's Hole, **110,** 201.

The Vascular System of Crustacean Compound Eyes, especially those of the Euphausid, *Meganyctiphanes norvegica*

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(*From the Marine Station, Millport, Isle of Cumbrae*)

With one plate (fig. 2)

SUMMARY

When the compound eyes of *Meganyctiphanes* and a number of decapods were examined a constant general pattern of blood-vessels was found.

The optic artery produced a branch to each of the following: the medulla terminalis, medulla interna, medulla externa, lamina ganglionaris, and the subretinal region. It then terminated in the dorsal region of the eye, passing the remaining blood into the ommatidial sinus.

The blood from the ommatidial sinus and from the systems of fine vessels in the nerve ganglia and subretinal region flows into the eye-stalk sinuses, whence it reaches the cephalothoracic sinuses.

INTRODUCTION AND PREVIOUS WORK

DURING work on the general morphology of *Meganyctiphanes norvegica* M. Sars, sections of the eyes were examined and a group of conspicuous cells', whose function was unknown, was found proximal to the basement membrane. The question arose whether these structures were peculiar to euphausids, and possibly the site of vitamin A synthesis, or were present in other crustacean eyes. Dr. T. H. Waterman (personal communication) had suggested they might be blood-vessels.

The eyes of *Meganyctiphanes* were then examined in detail and these structures identified in sections as sub-branches of the optic artery. The eyes of some other Crustacea were similarly investigated, but not in detail, to see whether there was a general pattern in the vascular system of compound eyes.

Blood-vessels are rarely mentioned in the numerous descriptions of compound eyes. The only relevant paper found is that of Mayrat (1956), which was published while the present work was in progress. He describes the vascular system in the eye of the mysid, *Praunus flexuosus* (O. F. Müller).

Hanström (1948) has reviewed the work done by himself and Carstam on the morphology of the eyes of *Meganyctiphanes*.

MATERIAL AND METHODS

The Crustacea, other than *Meganyctiphanes*, which were examined are as follows:

Macrura Natantia	*Leander squilla* (Linn.)
Macrura Reptantia	*Nephrops norvegica* (Linn.)
Anomura	*Eupagurus bernhardus* (Linn.)
	Eupagurus prideauxi (Leach)
	Galathea squamifera (Leach)

A solution of 50% carbon black VS paste was injected through very fine glass pipettes into the hearts of living *Meganyctiphanes norvegica*, *Nephrops norvegica*, *Eupagurus bernhardus*, and *E. prideauxi*. When the anterior arteries were full of carbon the heart-beat was arrested in 10% formalin in sea-water. The eyes were immediately severed from the specimens and dissected under glycerine, which served to prevent the nerve ganglia from becoming opaque.

Sections of *Leander squilla*, *Eupagurus* spp., and *Galathea squamifera* were examined, as also were sections of the eyes of *Meganyctiphanes* injected with carbon.

<div align="center">RESULTS</div>

Meganyctiphanes norvegica

In *Meganyctiphanes* the median aorta cephalica passes anteriorly and ventrally from the heart, supplying by branches the antennules, the cephalothoracic blood-gland, part of the stomach, and the brain; the main artery finally divides, at the base of the eye-stalks, into the two optic arteries, one to each eye.
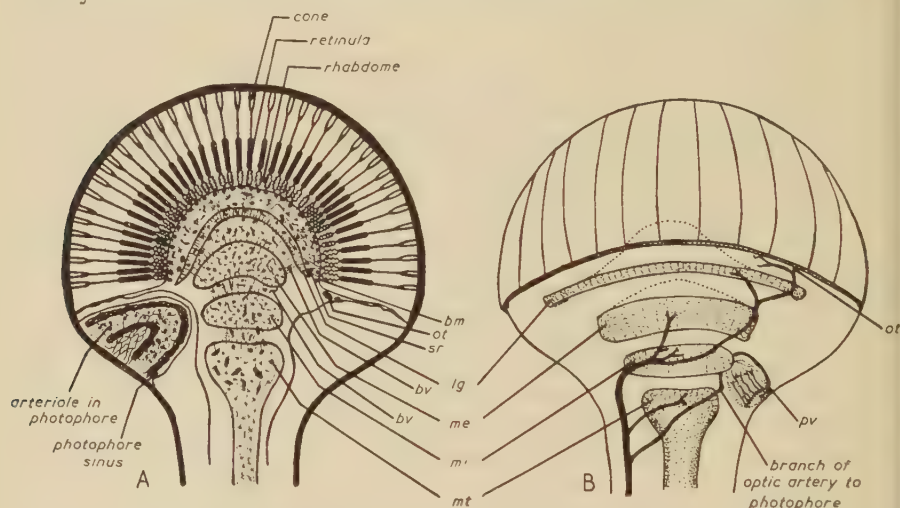

FIG. 1. A, a schematic drawing of a section of an eye of *Meganyctiphanes* showing the distribution of fine blood-vessels in the ganglia, the regions between the ganglia, and the photophore. B, a schematic drawing of an eye showing the course of the optic artery and its branches. *bm*, basement membrane; *bv*, blood-vessel; *lg*, lamina ganglionaris; *me*, medulla externa; *mi*, medulla interna; *mt*, medulla terminalis; *ot*, end of optic artery; *pv*, photophore branch divides into three; *sr*, subretinal arteriole.

The structure of the superposition eye of *Meganyctiphanes* is complicated by the presence of a photophore in the ventral region of the eye-stalk (the orientation of the eye is taken from its position in the animal). The first branch of the optic artery—which at first runs out in the inside lateral edge of the eye-stalk between the cuticle and the nerve ganglia—serves this photophore and, as far as can be determined, it alone. When this branch reaches the frontal edge of the light organ it divides in three (fig. 1, B, *pv*), the median
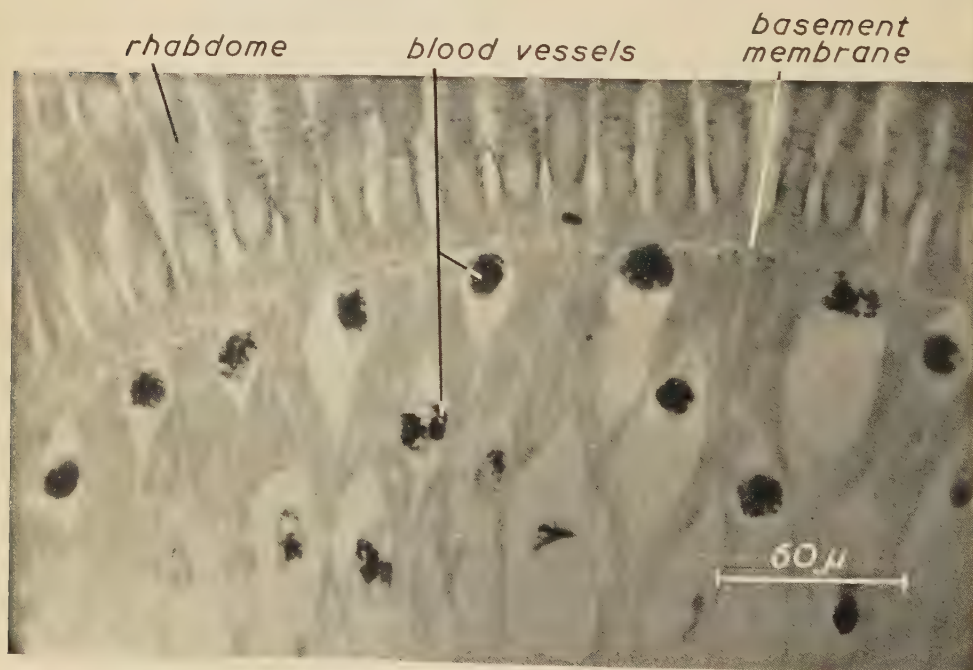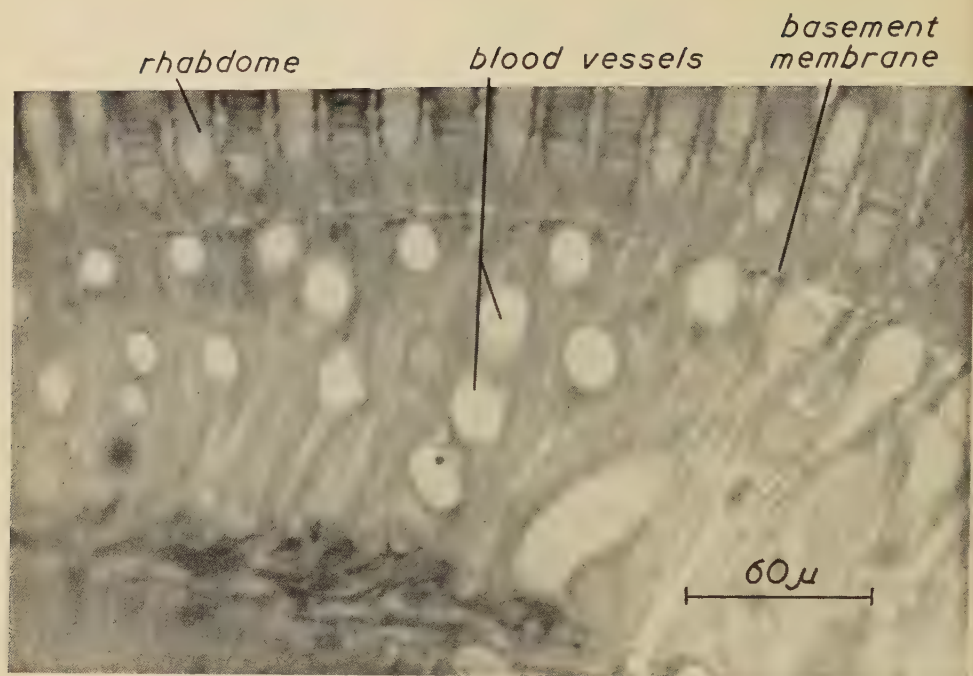
rhabdome   blood vessels   basement membrane

rhabdome   blood vessels   basement membrane

60μ

60μ

Fig. 2

J. MAUCHLINE

branch being very fine. The lateral branches produce numerous branches which ramify down through the 'posterior cellular layer' (Vallentin and Cunningham, 1888) and the outer edges of the striated body. The blood from these vessels enters the ocular sinuses through a sinus, the photophore sinus, which surrounds the sides and inner surface of the photophore (fig. 1, A).

The second branch from the optic artery supplies the medulla terminalis (fig. 1, B, *mt*) with a complex system of fine vessels which spread throughout its tissues (fig. 1, A, *mt*). A few fine sub-branches from it pass outwards to supply part of the medulla externa.

The main artery (fig. 1, B) then curves round the nerve ganglia towards the dorsal side of the eye, serving *en route* the medulla interna and medulla externa (fig. 1, B, *mi*, *me*). These two ganglia are very rich in blood-supply, the two systems being connected by vessels travelling across the intervening space in both directions.

The lamina ganglionaris (fig. 1, A, *lg*, *sr*) and the region of the eye between it and the basement membrane are each supplied by a sub-branch of the optic artery, which is now found in the dorsal region of the eye. A high concentration of blood-vessels is present among these nerve-fibres below the basement membrane.

Pressed close to the basement membrane is a layer of fine arteries which seem to present a constant pattern (figs. 1, 2); they run out round the membrane from the dorsal to the ventral side of the eye. This subretinal supply is extremely rich and it was at first thought that here was the source of nutrients for the ommatidia. Later, however, carbon was found in the spaces between the ommatidia, and the main optic artery was seen to end against the basement membrane. No blood-vessels were found distal to the basement membrane.

In *Leander squilla* also (see later) the optic artery ended against the membrane and an opening was found in the membrane at the end of the artery; the blood appeared to pass into the complex of cavities between the ommatidia (the ommatidial sinus). The same thing must take place in *Meganyctiphanes*.

In the ventral side of the eye there is a complex of sinuses between the photophore and the basement membrane. It is through these that the blood from the ommatidial sinus must gain access to the eye sinuses proper.

It is well known that there is an outer and inner eye sinus present in the eye-stalk, the two becoming one at the base of the eyes where the blood then enters the cephalothoracic sinus. The blood from the complexes of fine vessels is simply voided from their distal ends into the eye-stalk sinuses, whence it is finally returned to the heart.

Other species

In *Leander squilla* a similar pattern of vessels is found. The three medullas each have a ramifying system of fine vessels and so also has the lamina gan-

FIG. 2 (plate). Sections showing the rhabdomes, basement membrane, and subretinal region of the eye of *Meganyctiphanes*. In A the blood-vessels were not injected; in B they were filled with carbon black.

glionaris. Here again there is a very rich supply lying between the lamina ganglionaris and the basement membrane. No blood-vessels were found distal to the membrane. In serial sections the optic artery was seen to open through the basement membrane in the dorsal region of the eye, into the ommatidial sinus. In the ventral region of the eye a complex of sinuses, similar to that found in *Meganyctiphanes*, was seen. It is through these that blood from the ommatidial sinus reaches the outer eye-stalk sinus.

The eyes of *Nephrops norvegica* were examined by dissection only. A branch of the optic artery was found associated with each nerve ganglion and a rich subretinal supply was observed.

In *Eupagurus* spp. the ganglia were riddled with blood-vessels and an extremely rich subretinal supply was present. No blood-vessels were found distal to the basement membrane, though a large amount of blood was present in the ommatidial sinus. Again the ommatidial sinus opened into the outer eye-stalk sinus.

In the sections of *Galathea squamifera* a branch of the optic artery to each ganglion was found. The eye has a rich subretinal circulation and also a copious supply to the lamina ganglionaris. Again no blood-vessels were found distal to the basement membrane, though blood was found between the retinulae.

Conclusions

A constant pattern of blood-vessels is apparent in the eyes examined, a branch of the optic artery being associated with each of the three medullas and with the lamina ganglionaris. The final branching of the main artery supplies the groups of optic nerve-fibres proximal to the basement membrane and also produces a very rich subretinal layer of fine vessels. In all cases the artery terminates at the basal membrane, the remaining blood passing into the ommatidial sinus.

The blood from the ommatidial sinus and from all these systems of ramifying vessels flows into the eye-stalk sinus, whence it is returned to the heart through the gills.

If the above results are compared with Mayrat's for the mysid, *Praunus flexuosus*, a similarity is immediately apparent. His drawing shows more detail than fig. 2, but if this figure were made comparable to that of Mayrat the basic pattern would be obscured. He shows five main branches of the optic artery, supplying the nerve ganglia and the subretinal region, but has not found the main artery terminating in the basement membrane, which was found in the euphausid and the decapods examined here.

In *Meganyctiphanes* there is an extra branch, the one to the photophore, no comparable branch being present in any of the other Crustacea investigated.

REFERENCES

HANSTRÖM, B., 1948. 'The brain, the sense organs, and the incretory organs of the head of the Crustacea Malacostraca.' Bull. biol. France-Belgique, Suppl. **33,** 98–126.

MAYRAT, A., 1956. 'Œil, centres optiques et glandes endocrines de *Praunus flexuosus* (O. F. Müller).' Arch. Zool. exp. gén., **93,** 319–53.

VALLENTIN, R., and CUNNINGHAM, J. T., 1888. 'The photosphaeria of *Nyctiphanes norvegica* G. O. Sars.' Quart. J. micr. Sci., **28,** 318–41.

The Structure and Function of the Gas Bladder in *Argentina silus*

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With one plate (fig. 1)

SUMMARY

Anatomical and histological observations were made on the gas bladder of the soft-rayed marine salmonoid fish *Argentina silus*, which usually lives at depths of about 200 m. The gas bladder is completely closed but rather unlike other known types of closed gas bladders. It is composed of three tissue layers, which may conveniently be called mucosa, submucosa, and tunica externa. The latter contains a large amount of guanine. The tunica externa is penetrated by numerous blood-vessels, which form a widely distributed rete mirabile of a peculiar 'bi-dimensional' type in the submucosa. All vessels reaching the mucosa seem to come from this rete system, and no special 'resorbent mucosa' such as occurs in euphysoclistae could be identified. The mucosa is folded, forming an alveolar-like structure of unknown function. The gas gland layer is separated from the lumen of the bladder by smooth muscles and an inner epithelium. The close anatomical association of the gas gland tissue with smooth muscles probably is of importance for gas secretion. Analyses of gases from specimens taken from a depth of about 80 m showed a mean oxygen value of almost 80%, which indicates a similar gas secretory mechanism to that in euphysoclistae. Certain aspects of the physiology of the gas bladder in relation to life in deep water are shortly discussed, and the need for a thorough histological examination of the gas bladder in deep-water salmonids is emphasized.

INTRODUCTION

FISHES living below depths of 500 to 1,000 m usually have no gas bladder or have this converted into a fat storage organ (Marshall, 1954). Obviously below a certain depth inflation of a gas bladder is physiologically impossible owing to the water pressure. So far as deep-sea fishes have a functioning gas bladder, this is of the closed type. Even among the predominantly physostomatous order Isospondyli the deep-living forms have no pneumatic duct (Jones and Marshall, 1953). An exception are the deep-living fresh-water salmonids which have a pneumatic duct. As may be expected, the gas bladders in deep-sea fishes show certain anatomical features which might be considered as adaptations to their environment. Thus in Gonostomatidae, Sternopty-chidae, and Myctophidae the gas gland and the rete mirabile are larger than in surface-living species (Marshall, 1954).

The present investigation has been devoted to the gas bladder of *Argentina silus* (suborder Salmonoidea of the Isospondyli). This fish lives at depths between 200 and 550 m 'along the edge of the continental platform' (Murray and Hjort, 1912). It is known to have a closed gas bladder (de Beaufort, 1909;

Jones and Marshall, 1953), but the more detailed anatomy of this organ does not seem to have been studied before.

<center>MATERIAL AND METHODS</center>

The material consisted of five small specimens (body length 28–30 cm) taken by trawling from a depth of about 80 m, which is unusually shallow for the species. At the time of capture the gas bladder had exploded in two specimens but was apparently undamaged in the other three. The fishes were all dead or moribund. Samples of gases were taken immediately after the fishes were caught and were analysed on board the investigation ship. For the analyses the Krogh micro gas analysing apparatus was used (Fänge, 1953). Dissections were made on fresh, unfixed specimens. For histological examination the gas bladders or parts of them were fixed in Bouin's fluid. Paraffin sections were stained with Azan or with haematoxylin and eosin.

<center>RESULTS</center>

Gross anatomy

The gas bladder is slender and spindle-shaped (fig. 1, A). In the specimens investigated (not fully grown) the length of the bladder was 10–14 cm. Beneath the peritoneal cover the bladder has a silvery, shining appearance. The organ is almost perfectly circular in transverse section.

Many blood-vessels pass to the gas bladder from the region of the large dorsal vessels (fig. 2, B). Six to ten large vessels were seen on each side. It was not possible to determine which of these vessels were arteries and which were veins, because the soft, oily consistency of the body-tissues made macroscopic studies difficult. No connexion could be observed between the gas bladder vessels and the hepatic portal system. The blood-vessels reaching the bladder branched over its surface (fig. 2, B), and the branches passed into the interior of the bladder-wall through numerous small transverse 'clefts'. The internal surface of the bladder was folded in a characteristic manner, forming alveolar-like structures somewhat reminiscent of a honeycomb (fig. 2, A). The folds were restricted to the mucosa and did not involve the external parts of the bladder-wall.

FIG. 1 (plate). A, the isolated gas bladder. Note the absence of pneumatic duct and the metallic appearance due to guanine. The numerous small 'clefts' are entrance places for blood-vessels.

B, transverse section through the bladder-wall. In the submucosa numerous rete mirabile bundles are seen. The tunica externa has a striated appearance. (Haematoxylin-eosin.)

C, surface preparation of the unstained mucosa. Winding capillary loops are seen. A few rete mirabile bundles are indistinctly visible (upper left).

D, longitudinal section showing a mucosal fold belonging to the alveolar-like structure of the mucosa. The muscularis mucosae at this place consists mainly of circularly arranged fibres. (Azan.)

E, transverse section. In the lower part of the figure submucosa is seen, with several rete mirabile bundles. (Azan.)

F, transverse section. At this place the muscularis mucosae contains both circularly and longitudinally arranged smooth muscle-fibres. The glandular layer is poorly developed and indistinctly seen. (Azan.)
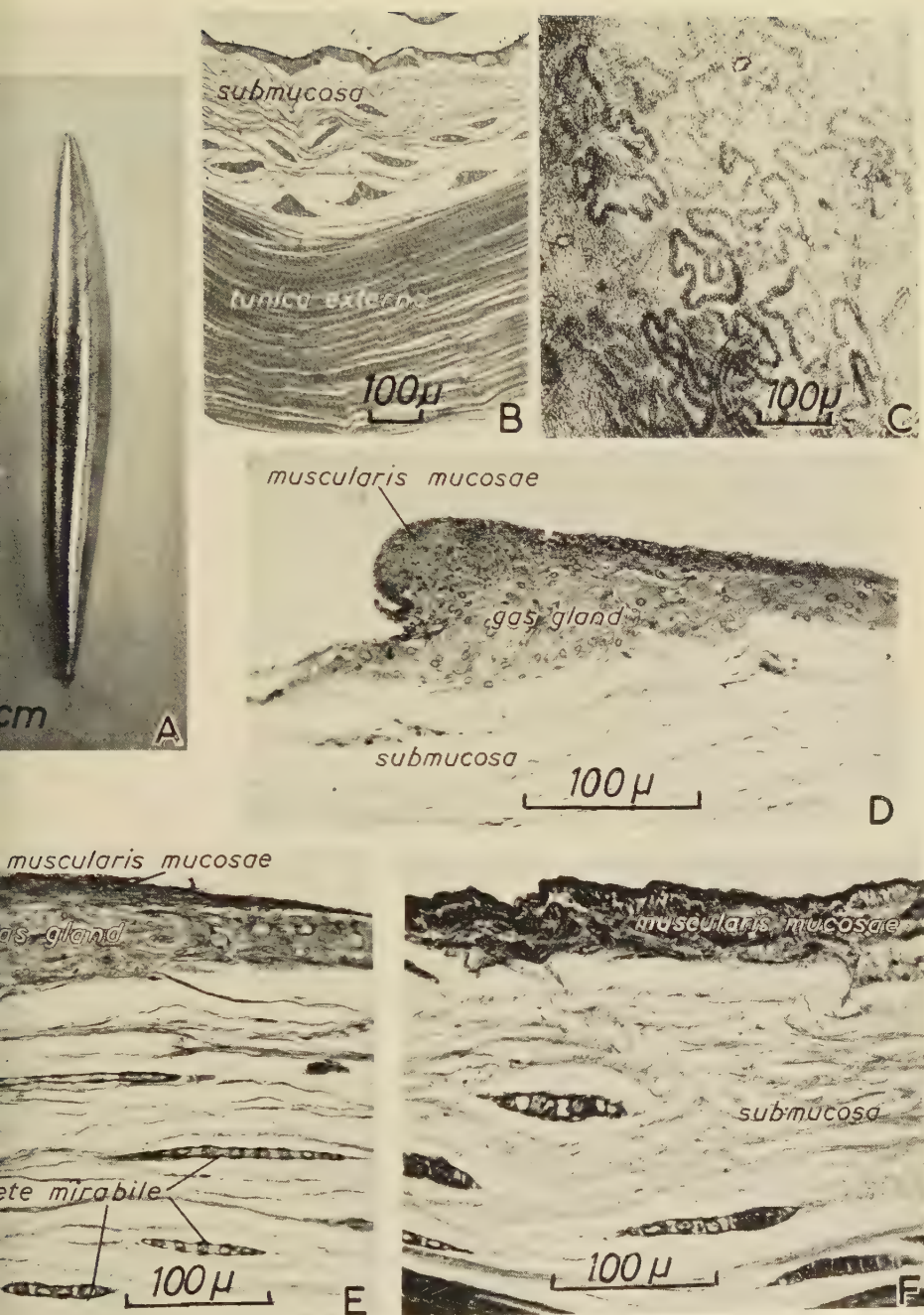
FIG. I

R. FÄNGE

Microscopic anatomy

It is often possible to distinguish three layers in the wall of the gas bladder: tunica externa, submucosa, and mucosa (Fänge, 1953; O'Connel, 1955). In *Argentina* these layers stand out very clearly. Outside the tunica externa there is a ventral peritoneal cover; thus the gas bladder is situated retroperitoneally.
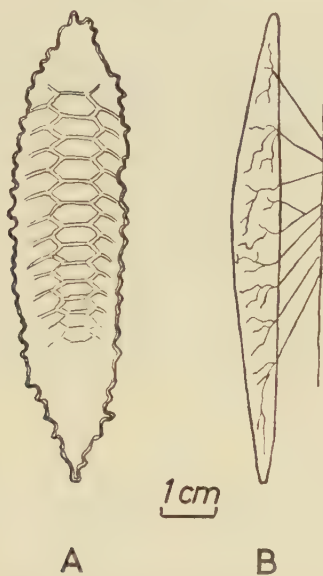

1 cm

A   B

FIG. 2. A, the inside of the gas bladder showing the honeycomb-like appearance of the mucosa. B, sketch of the gas bladder, left side view, showing blood-vessels reaching the bladder from the region of the dorsal aorta and the cardinal veins.

The tunica externa. The external two-thirds of the bladder-wall consist of a dense connective tissue layer probably corresponding to the tunica externa of other species. Immediately below the peritoneum the tunica externa has a loose fibrous texture and contains large blood-vessels mainly longitudinally arranged. The major part of the tunica externa consists of a dense lamellar connective tissue loaded with guanine. The guanine lamellae run circularly more or less like barrel-hoops in the bladder-wall. At rather regular intervals (30–100 μ apart from each other) blood-vessels pass through the tunica externa perpendicularly to the connective tissue lamellae. The penetrating blood-vessels are surrounded by their own connective tissue sheaths consisting of collagenic membranes. The heavy deposit of guanine in the gas bladder of *Argentina* has previously been mentioned by Voit (1865), according to whom guanine crystals from the gas bladder of *A. sphyraena* have been used to manufacture pearl essence for making artificial pearls. Probably owing to an abundance of guanine lamellae the tunica externa in histological sections has a characteristic striated appearance (fig. 1, B). The occurrence of guanine in the wall of the gas bladder is also known from other fishes, for instance clupeids (Maier and Scheuring, 1923).

H

The submucosa. This is a layer of very loose connective tissue situated between the tunica externa and the mucosa. In this layer there occur numerous ribbon-like bundles of small blood-vessels (fig. 1, B, E, F). They are oriented mainly in a longitudinal direction. Each bundle consists of a single row of alternating arterioles and venules. The bundles constitute a sort of rete mira-bile which is described further on in the text.

The mucosa. The mucosa consists of a very thin inner epithelium, a smooth muscle-layer ('muscularis mucosae'), and a glandular layer. The muscularis
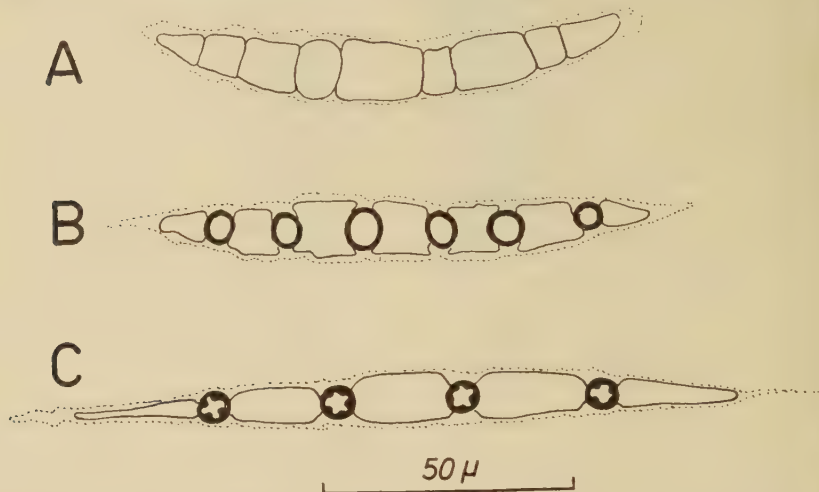

FIG. 3. Different shapes of the rete mirabile bundles. In A the arterioles and venules are almost indistinguishable from each other. In B and C the arterioles stand out by their thicker walls, probably containing smooth muscle elements. The dotted line indicates a layer of connective tissue cells surrounding the rete mirabile bundles. (Camera lucida drawing.)

mucosae is composed of bundles of smooth muscles arranged both circularly and longitudinally. At certain places the smooth muscle-layer is thickened, forming the alveolar structures mentioned above (fig. 2, A). The glandular layer is richly vascularized by winding capillary loops coming from the rete mirabile bundles (figs. 1, C; 4). The glandular cells are irregularly shaped and often look more like modified connective tissue cells than epithelial cells. They are often binucleate, and some of the cells are very large and contain deformed or fragmented nuclei. Their cytoplasm is finely granulated and stained reddish by Azan. Often the cytoplasm is highly vacuolated (fig. 4). In its general appearance the glandular layer resembles to a considerable extent the glandular layer of the gas bladder in cyprinids (Fänge and Mattisson, 1956). Like this it is separated from the lumen of the gas bladder by a smooth muscle-layer and the inner epithelium.

The rete mirabile. The bi-dimensional arrangement of the vessels composing the rete mirabile is typical of *Argentina* and has hitherto not been observed in any other fish, although the caudal part of the rete mirabile in the cyprinid

gas bladder has a somewhat similar structure. Alternating arterioles and venules are situated close together in single rows. Such flat bundles usually consist of 6 to 10 vessels but as many as 16 to 18 may be counted, while the smaller bundles are composed by only two vessels—a small artery and its
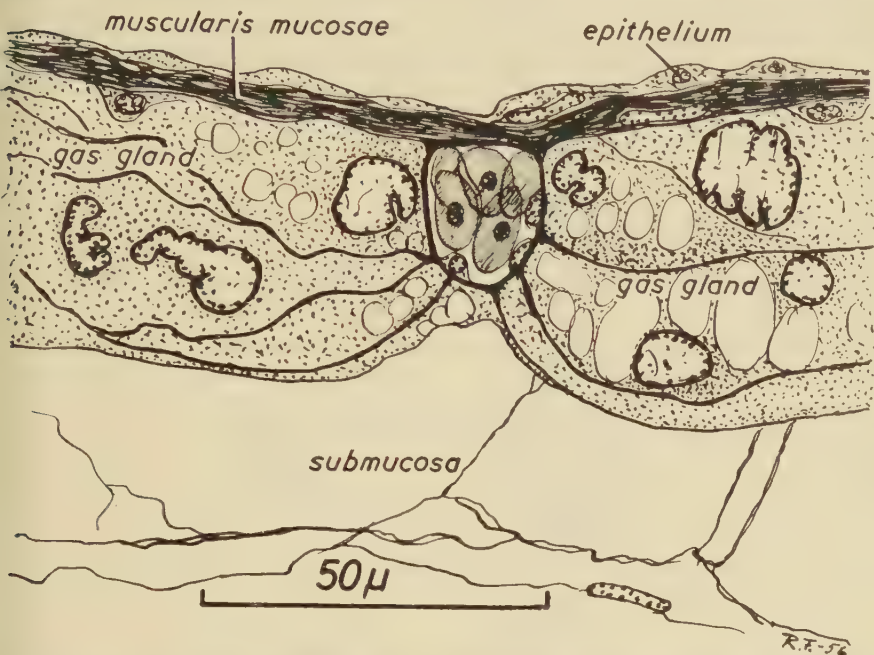

Fig. 4. Part of the mucosa of the gas bladder, in transverse section. (Camera lucida drawing.)

accompanying vein. The two-vessel bundles are formed by successive dichotomous division of larger bundles. At their distal ends the two-vessel bundles continue as capillary loops winding in the glandular layer (figs. 1, c; 5).

The arterioles of the rete mirabile have a varying appearance. In some bundles they have thin walls and are almost indistinguishable from the adjacent venules (fig. 3, A). In other bundles the arterioles have thick walls (fig. 3, B), and often their endothelium is folded with nuclei protruding into the arterial lumen (fig. 3, C). The latter structure might indicate that the arterioles are able to contract and shut off the rete circulation by action of their smooth muscles. The rete mirabile bundles containing thick-walled arterioles are for the most part situated near the tunica externa and the bundles containing thin-walled arterioles near the mucosa. It may be recalled that supposedly contractile arterioles are found in the proximal part of the rete mirabile of euphysoclistae (Fänge, 1953, p. 30). Those parts of the rete mirabile where the arterioles have thin walls are certainly better suited for diffusion processes than those containing thick-walled arterioles.

In a transverse section 8μ thick through the anterior part of the gas bladder, which is 5 mm thick at this point, 144 bundles were counted and in another

similar section 122 bundles. If we assume a mean of 10 blood-vessels in each bundle, there are thus more than 1,000 individual vessels cut in a single histological section. Now blood-vessels penetrate the tunica externa along the whole length of the bladder, giving rise to rete mirabile bundles in the underlying submucosa. Thus the number of over 1,000 has to be multiplied by a factor of perhaps about 50 (the exact number can only be found by recon-struction studies) in order to give the absolute amount of individual blood-vessels participating in the rete mirabile. The value 50,000 arrived at by this
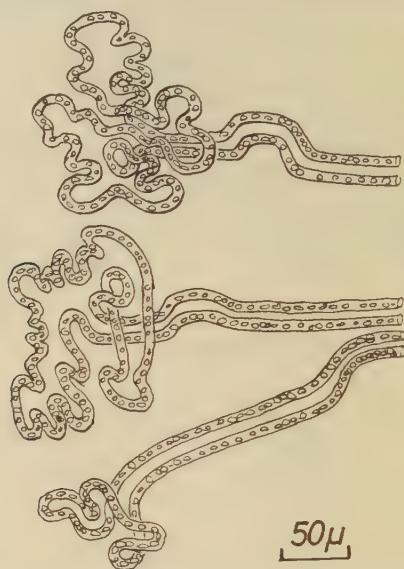

FIG. 5. Three capillary loops in the mucosa drawn from a fresh unstained specimen.

rough calculation is of course very uncertain, but it is interesting that it is similar to that calculated by Krogh (1936) for the well-developed rete mirabile of the eel (196,000 individual blood-vessels).

The composition of the gases contained in the bladder

As a rule the gas bladder of fishes living in deep water contain a high per-centage of oxygen. That this is also true for *Argentina* is shown by the values from three specimens given below:

No.	Carbon dioxide, %	Oxygen, %
1	0·8	74·7
2	0·8	77·7
3	0·0	79·8
Mean values	0·5	77·4

DISCUSSION

The results from the gas analyses show that in the closed gas bladder of *Argentina* there occurs a gas secretion similar to that in euphysoclistae,

producing a gas mixture rich in oxygen. In all fishes which are able to inflate their bladders by so-called gas secretion, there are vascular specializations of rete mirabile type. Even in cyprinids, where gas secretion is a very slow process, there are rete mirabile formations (Fänge and Mattisson, 1956), although composed of few vessels. In *Argentina* the rete mirabile is of an uncommon type, but it consists of many thousands of vessels and obviously has a large functional capacity. Most probably a rete mirabile is a necessary equipment for the process of gas secretion. (For a discussion of its role see, for instance, Maetz, 1956.) Scholander (1954) showed that the rete mirabile provides a counter-current diffusion barrier against oxygen loss through the blood leaving the bladder.

In euphysoclistae there can be distinguished two parts of the mucosa, a secretory part and a resorbent part, each with its own type of circulation. The secretory mucosa is furnished with blood from the rete mirabile while the resorbent mucosa is vascularized by a non-rete system. In *Argentina* all the blood supply to the mucosa probably comes from the rete mirabile bundles. This must provide a good protection against losses of oxygen from the bladder. Certainly the wide distribution of rete mirabile bundles in the submucosa of the *Argentina* gas bladder is a special adaptation to deep-sea life. The function of the alveolar-like structure of the mucosa is enigmatic. It may be mentioned in this connexion that similar alveolar-like mucosal structures have been observed in certain other fishes belonging to the order Isospondyli (*Megalops, Engraulis*, &c.) (de Beaufort, 1909). The abundant guanine lamellae in the tunica externa perhaps gives protection against the disappearance of gases from the bladder.

Whereas *Argentina*, like other deep-water marine fishes, has a closed gas bladder, the coregonids that inhabit fresh water are physostomatous. The deep-water coregonids are peculiar in that they have a very low oxygen content in their gas bladder (Hüfner, 1892; Scholander, 1956).

No gas gland or rete mirabile has been observed in *Coregonus* and other salmonids, but Corning (1888) observed that the anterior part of the gas bladder in *Salmo* is richly vascularized near the entrance of the pneumatic duct. Perhaps a thorough histological examination of the gas bladder in salmonids would reveal at least in certain species the existence of rete mirabile bundles reminiscent of those in *Argentina*, although less numerous. The observation by Saunders (1953) that in *Coregonus clupeaformis* taken from shallow water the gas bladder occasionally contains as much as 42·3% of oxygen strongly suggests that there exists in salmonids a gas secretory mechanism not greatly unlike that of other fishes. But how can the high nitrogen percentage and the low oxygen percentage in the gas bladder of coregonids taken from deep water be explained? Powers (1932) has proposed a theory according to which gases are added in small quantities to the bladder by means of bubbles, independently of the partial pressure in the gas bladder. Owing to their greater diffusibility through living membranes, carbon dioxide and oxygen are more easily reabsorbed by the blood than the inert gases.

According to the author's observations bubbles are formed in clefts and ducts of the gas gland in *Ctenolabrus, Spinachia*, and other euphysoclistae during gas secretion (Fänge, 1953). However, no clefts or ducts are known from the gas bladder mucosa of such fishes as salmonids, cyprinids, and *Argentina*.

In *Argentina* there is an intimate association between the gas gland tissue and the smooth muscle-layer (muscularis mucosae). Such a close association of gas gland tissue with smooth muscles is of general occurrence and has been found in many different fishes (Fänge and Mattisson, 1956). Probably it is of importance for gas secretion, because this seems always to be correlated with relaxation of the smooth muscles. One possibility is that the movements of the muscularis mucosae could have a mechanical effect upon the release of newly formed gas bubbles into the bladder. Hagman (1952, personal communication) has used hot mercuric chloride fixative for the demonstration of gas bubbles in active gas glands. Unfortunately he died without publishing his results. Certainly his technique would be of value for further studies of the process of gas secretion.

REFERENCES

BEAUFORT, L. F. DE, 1909. Morph. Jb., **39,** 526.

CORNING, H. K., 1888. Morph. Jb., **14,** 1.

FÄNGE, R., 1953. Acta physiol. scand. **30,** Suppl., 110, 1–133.

—— and MATTISSON, A., 1956. In Zoological Papers dedicated to Bertil Hanström, 153–62.

HAGMAN, N., 1952. Personal communication.

HÜFNER, G., 1892. Arch. Anat. Physiol., physiol. Abt. (no vol. number), **54.**

JONES, F. R. H., and MARSHALL, N. B., 1953. Biol. Rev., **28,** 16.

KROGH, A., 1936. *The anatomy and physiology of capillaries.* New Haven (Yale University Press).

MAETZ, J., 1956. Bull. biol., Suppl., **40,** 1–148.

MAIER, H. N., and SCHEURING, L., 1923. Wiss. Meeresunters. Abt. Helgoland, **15, 1.**

MARSHALL, N. B., 1954. *Aspects of deep sea biology.* London (Hutchinson).

MURRAY, J., and HJORT, J., 1912. *The depths of the ocean.* London (Macmillan).

O'CONNEL, C. P., 1955. Fishery Bull. Fish and Wildlife Service, **56, 503.**

POWERS, E. B., 1932. Amer. J. Physiol., **101,** 84.

SAUNDERS, R. L., 1953. Canad. J. Zool., **31,** 547.

SCHOLANDER, P. F., 1954. Biol. Bull., **107,** 260.

——, VAN DAM, L., and ENNS, T., 1956. Science, **123,** 59.

VOIT, C., 1865. Z. wiss. Zool., **15,** 515.

Integration in Aggregating Cellular Slime Moulds

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With four plates (figs. 1-4)

SUMMARY

1. Cellular-slime-mould amoebae that adhere poorly to one another on contact and effectively do not secrete the chemotactic agent acrasin may become strongly adhesive and start to secrete it. This change, which is particularly important for their aggregation, has been called *integration*, and the reverse change, *disintegration*.

2. In *Polysphondylium violaceum* a cell may receive the integrative stimulus at some distance from the acrasin source that attracts it, but usually it does not change till it has moved close to it or actually reached it; so either an aggregation is simply a heap without any tributary cell-streams, or virtually continuous streams are slowly built out from the centre.

3. In *Dictyostelium discoideum* the spread of integration, in comparison with cell velocity, may be so rapid that the inflowing streams very soon reach their ultimate extent, though if the population density is not too high their cells only slowly establish contact with one another.

4. As the peripheral cells become integrated, they 'relay' the centre's influence. Whether the streams are uninterrupted or 'stippled', there need be no continuous and finely graded centrifugal decrement in acrasin secretion: orderly aggregation is ensured by the sequence in which secretion is induced, which results in the centrifugal propagation of one or more comparatively narrow zones in which the gradient is adequate for orientation.

5. A spontaneous or an experimentally produced decrease in the strength of the integrative stimulus, or adaptation to the stimulus, or both, may induce some integrated cells to revert to the unintegrated state.

6. The spread of *dis*integration, too, in comparison with cell velocity, tends to be more rapid in *D. discoideum* than in *P. violaceum*: in the former species all the cells in a considerable length of stream may begin to separate at almost the same time; in the latter they may detach themselves in succession.

7. As disintegration may affect any part of an aggregation, various patterns result therefrom. If it slowly spreads from the centre of a *P. violaceum* aggregation while the streams are still growing at their outer ends, a 'fairy ring' is formed, in which cell movement remains polar.

8. Populations of a *D. mucoroides* strain, if not too sparse, aggregate at a comparatively fixed time after consuming the available food; but those of *D. discoideum* at widely different densities and degrees of starvation produce synchronous outbursts of aggregation when transferred from darkness to light.

9. The development and distinctive properties of the initiators of aggregation centres are considered. These cells can release acrasin into the medium without there being any there already or without there being sufficient to induce the remaining cells to secrete.

10. Much evidence is against aggregation being basically a sexual phenomenon.

Contents

INTRODUCTION

THE post-vegetative amoebae of the cellular slime moulds *Dictyostelium* and *Polysphondylium* flow in *streams* towards collecting *centres*; this aggregation is preparatory to building aerial fruiting bodies (Raper, 1940*a*, *b*, 1941). Specific chemotaxis is one of the factors involved (Bonner, 1947; Shaffer, 1953, 1956). In the earlier papers in this series (Shaffer, 1957*a*, *b*), a distinction has been drawn between *integrated* and *unintegrated* cells: the former can adhere strongly to one another and usually secrete the chemotactic agent acrasin; the latter adhere but poorly and (effectively, at least) do not secrete it. In the present paper the transformations of cells from one state to the other are examined; these have been called *integration* and *disintegration*. The development of the first integrated cells, on which the initiation of aggregation depends, is considered in a separate section.

The organisms were cultured and operated on as previously described (Shaffer, 1957*a*). They were allowed to aggregate on saline agar at a sufficiently low cell-density for the cells to be well separated from one another before aggregation began. In certain specified instances they aggregated on glass under water (Bonner's technique, 1947).

All the plates are photographs of living cultures.

INTEGRATION

(i) In *Polysphondylium violaceum*

Frequently an aggregation in *P. violaceum* began with the cells piling themselves up into a relatively large centre and then grew by the progressive outwards extension from it of *continuous* streams (Fig. 1) although cells sensitive to acrasin might be found far beyond its edge. This suggested that contact with cells that were already integrated—those in centres and streams —was the usual stimulus that made unintegrated cells become integrated ones.

FIG. 1. *P. violaceum.* Scales represent 200 μ. A, streamless centres, recently formed, irregularly distributed, and of varying size. Two very small ones arrowed. B, growth of nearly continuous streams outwards from centres. C, D, further growth of aggregations
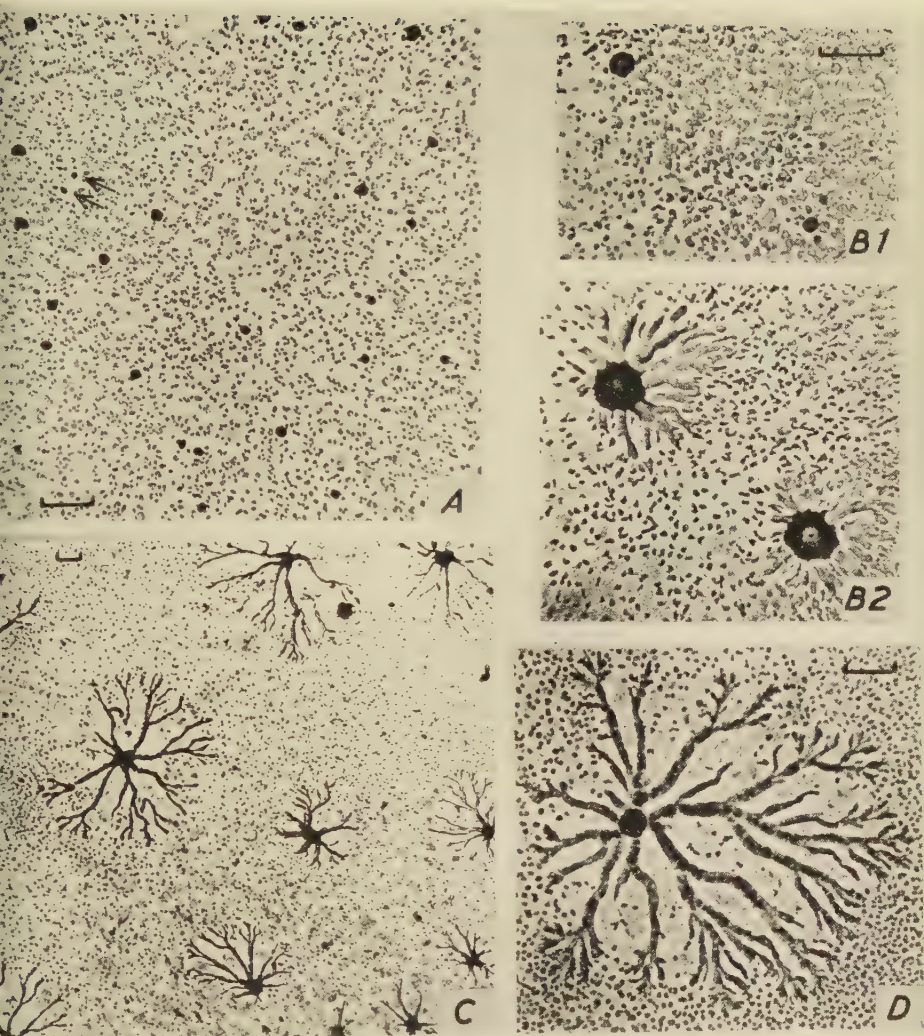
n alternative possibility was that the cells were stimulated to change while
ome distance away from the structure that attracted them, but that they
ther took as long to develop new properties as to crawl to it or were too far
part for any change that had taken place to be revealed on their way there.
Separate sensitive cells, either unaggregated or de-integrated, were swept
ith ball-tipped glass needles into two heaps of diameter about 100 to 150 μ.
One heap served as a control: its cells started to disperse immediately, and
sually within 10–15 min it was replaced by a patch of separate unoriented
ells. Thus, by itself, extensive contact between sensitive unintegrated cells
as insufficient to induce their integration. A young fruiting body, which
mitted acrasin, was deposited near the second heap and then carefully
ragged away as cells from the heap advanced towards it. (If its slime sheath
as not disrupted, few of its own cells were left behind, and these were easily
rushed aside.) By this operation the cell heap was induced to transform into
compact stream without its making contact with the fruiting body; and the
crasin secretion of this stream was attested by the reaction of the heap-cells
hat had been out of range of the original source and had begun to disperse.
f the population density was such that the separate sensitive cells were initi-
lly close together, a similar result could be obtained without the necessity
f sweeping them into heaps; in this case, the fruiting body was dragged over
n area first cleared of cells.

i) In *Dictyostelium discoideum*

In an experiment designed to find out whether amoebae could be attracted
y a centre without being connected to it by a continuous interface, Bonner
1947) mounted two coverslips as shelves side by side under water; one was
overed with amoebae that were just starting to aggregate, and on the other,
ery near the adjacent edge, there was a centre. This did attract the amoebae
n the first slip; and as they moved towards it, they joined up into streams,
ven if they were unable to cross the gap and make contact with it.
This experiment has given a similar result when performed with acrasin-
ensitive test cells that were not already aggregating, and it has thus provided
xcellent evidence that a source can induce integration at a distance. More
ecently (Shaffer, 1956) integration has been induced by acrasin solutions in
he absence of a living source.
Acrasin's induction of acrasin secretion complicated the bio-assay of its
mission (Bonner, 1949; Shaffer, 1957*a*); for though the orientation of cells
ttracted towards a source did initially record the direction of the maximum
radient of the chemical it produced, subsequently, unless they had already
eached it, they were liable to deflect one another when they themselves began
o secrete. When the differential in emission along a single source was estimated
y recording the extent to which the orientation of acrasin-sensitive indicator
ells diverged from the normal to it, the error due to induced secretion could
e minimized firstly by placing these cells close to the source and secondly
y depositing them not in a continuous streak but in a row of small heaps

that were as far apart as was practicable. When the acrasin emission of two
sources was compared by observing the partition of sensitive cells between
them, induced secretion might lead to the displacement of the zero-gradient
zone towards one or other of the sources unless these were very near together;
to reduce the error in this situation the cells were not distributed at all dis-
tances between the sources but were grouped in a narrow band distant from
one of them by some arbitrary fraction of the gap separating them.

When the preaggregation cells of *D. discoideum* were not too close together,
primary aggregation was marked by the nearly uniform orientation of many
of the cells over a wide area before they had made contact with one another.
Sometimes small centres were clearly visible before appreciable orientation
had occurred (fig. 2 A); sometimes they were not. The extent of the field
oriented within one system varied with the distribution of the centres and the
responding cells and the time intervals over which centres appeared: it might
be centimetres across. An aggregation looked as if it had been stippled with
short oriented strokes instead of drawn with solid lines; and because of the
distances involved, it was clear that orientation could not have been brought
about by the secretion of the centre alone, even if the cells, which in reality
were in various stages of developing acrasin sensitivity, had all been as sensi-
tive as those taken from the continuous streams that appeared later (the normal
indicator cells used in tests). That acrasin was secreted by the periphery was
made visibly manifest if the feathery expanses of cells led to the centre not
directly but in sweeping curves (fig. 2 B), which reflected the approximate
orientation of most of the separate cells within them. Two adjacent expanses
within the same aggregation might be almost mirror images of each other, and
thus cells on either side of the boundary between them might point in opposite
directions. In the extreme case, cells could be guided to a centre through a
pathway, formed by other cells, that was a nearly complete circle and first
led them straight away from it (fig. 2, C). Evidently the oriented cells, though
still separate, were already integrated.

In a stippled aggregation, the cells joined up in twos, threes, and half-
dozens to form fine, irregular, and disconnected threads (fig. 2, A), many of
which, judged over a small enough area, were roughly parallel, though devia-
tion could readily be found. The bands of cells visible at an early stage
condensed into compact streams in much the same positions; and the larger
systems fragmented into a number of smaller and more definite ones.

Fig. 2. *D. discoideum*. Scales represent 200 μ. A, growth of stippled aggregations. *3*, *4*, an area
near the centre of the main aggregation: note contractile vacuoles. Approximately $\frac{1}{2}$-h intervals
between *1* and *2*, *3* and *4*, *2* and *5*. Careful comparison between *2* and *3* and between *4* and *5*
reveals the finer details of how the cells join up into threads and streams; exposure intervals
approximately 1 min. *1*, *2*, *5*, some cells near the bottom are oriented to the right rather than
radially. *4*, the front of one streamlet has made a right-angled turn towards the one ahead of it,
while the latter has behaved in a similar way. *5*, the centre of the main aggregation has frag-
mented and one of the centres on the right transformed into a short length of stream. B, part
of a stippled aggregation, its centre being beyond the right-hand edge of the field. Some cells
on the left point upwards. C, aggregation more advanced and at a higher density. Some cells
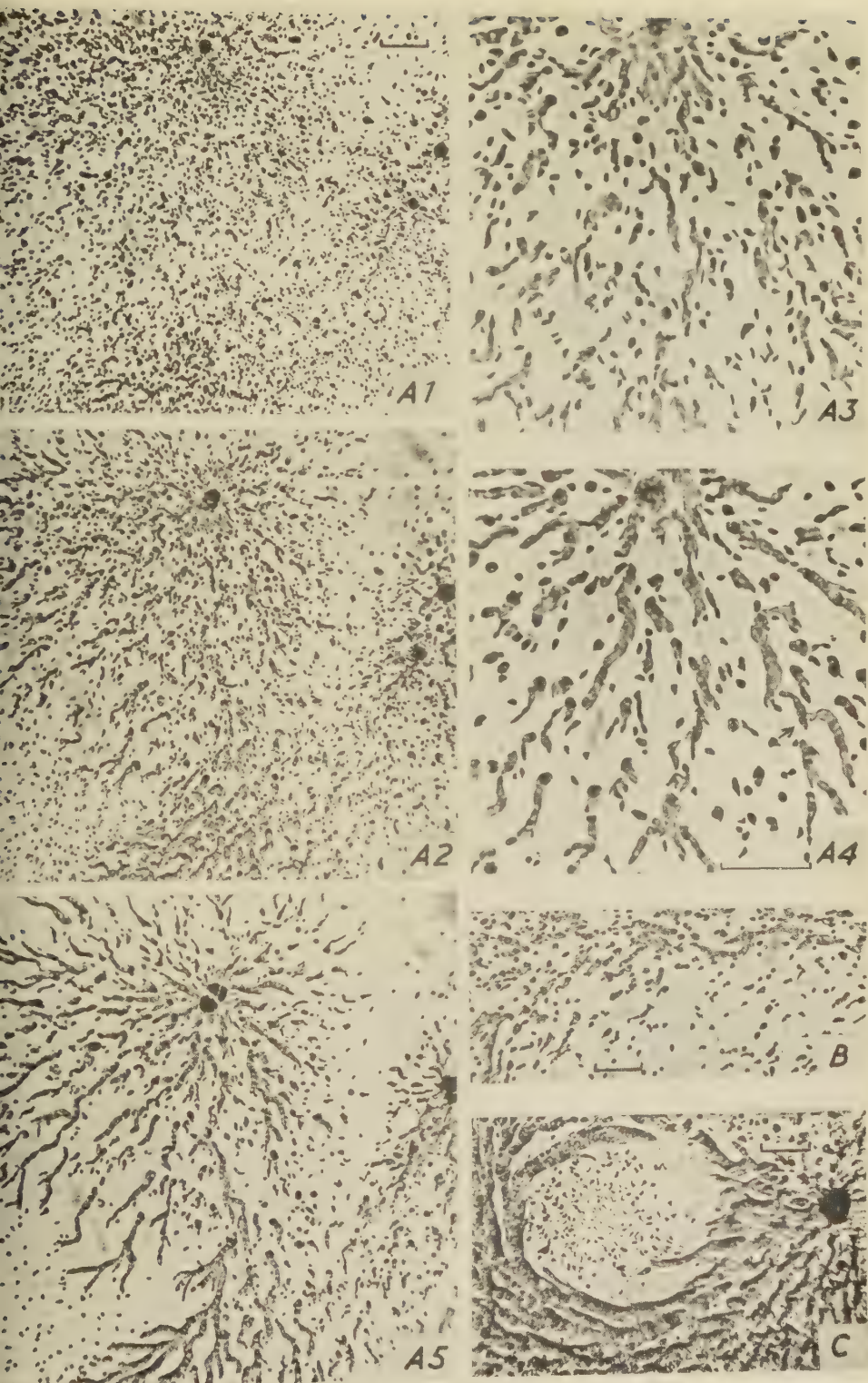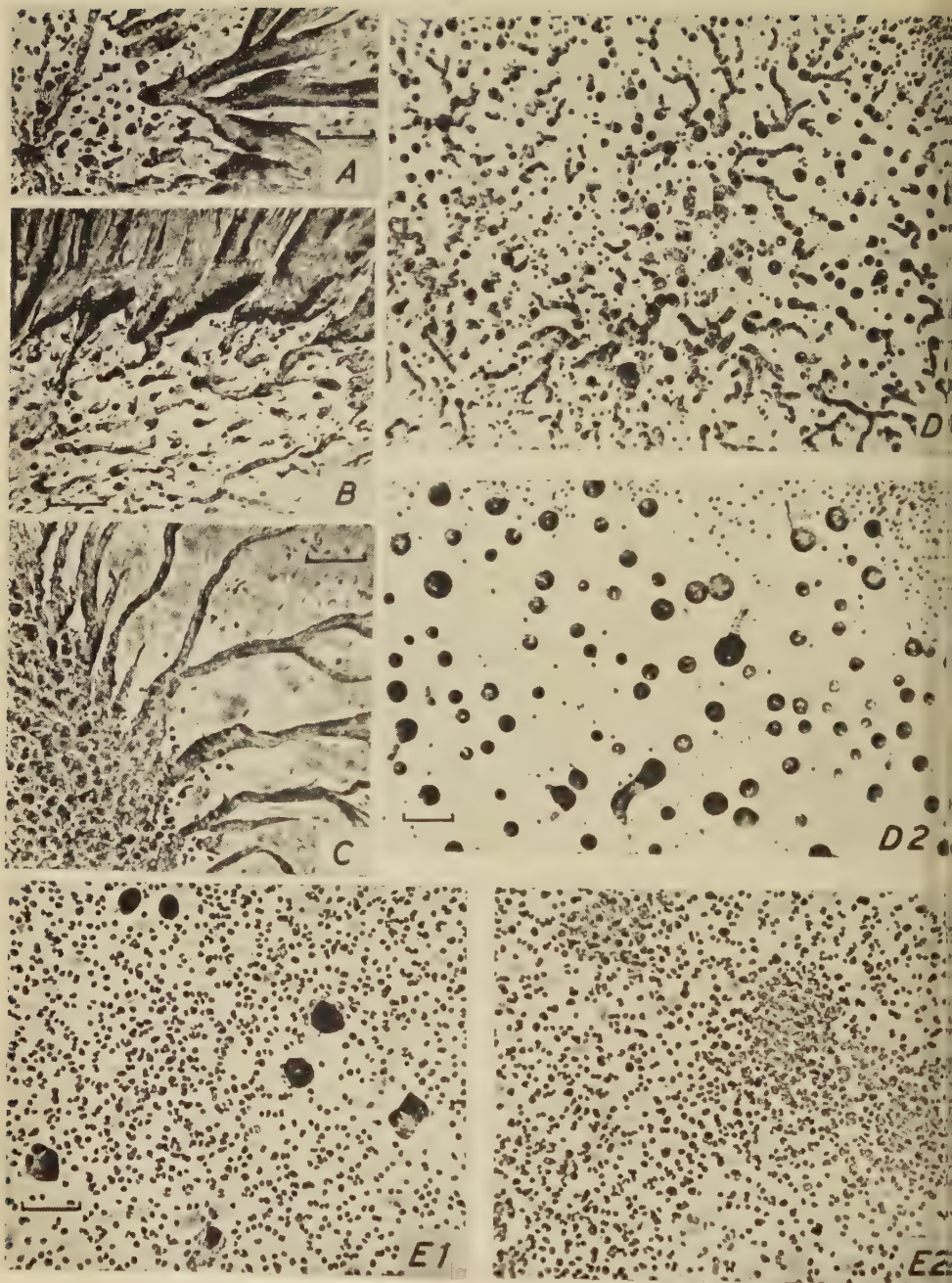point directly away from the centre

FIG. 2

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FIG. 3

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In one part of an aggregation the streams might be broad and almost con-
tuent ribbons, in another only tenuous strings; and as was particularly
obvious when the population was comparatively sparse, one area might have
the general appearance that another had had 1–2 hours earlier. Such differ-
ences in cell density or in progress of association, which were due to local
variations in the environment, including the relative amount of bacterial food
available earlier, might occur in the same sector: massive streams might feed
thin ones, and (figs. 3, A–C) considerable lengths of uninterrupted stream,
broad or narrow, be separated from the centre by an area of short discon-
tinuous threads or of cells that showed very little trace of association (but had
nonetheless relayed the acrasin stimulus).

The acrasin secretion of the cells in a stippled aggregation was tested by
one of Bonner's (1949) methods. Quick-moving, highly sensitive cells taken
from a more advanced aggregation were deposited in a bare, or bared, sector,
close to a radius of oriented cells: they crawled perpendicularly towards it.
It was concluded that, as with compact streams, there might be no steady
spatial differential in acrasin emission. Yet the separate cells that composed
the radius did not turn sharply towards the secreting cells on *their* flank, but,
in the main, preserved their existing orientation. On the other hand, if the
cells in a stippled aggregation were swept away early enough from an area
that extended radially about $250\,\mu$ and was several times as wide (cf. the cut-
ting of a continuous stream), cells in the outer part of the mutilated sector
might then turn towards the intact sectors on either side of them, though of
course these sources had been there all the time, and form concentric arcs
at right angles to their previous alignment. In a variation of this experiment,
the cells swept up were not removed but were used to build a radial bridge
across the bared swath. The cells farther out might then converge on the
outer end of this bridge, as if it were the centre, and in due course cross over
it. (If the bridge was built of cells from older aggregations, it was ineffective
in conducting the aggregative stimulus, unless replacements were added,
because the cells moved so rapidly towards the centre that they soon ceased
to span the gap.)

Thus it appeared that the orientation of these organizations was a record
of the spread of the aggregative stimulus and also tended to be self-preserva-
tive.

FIG. 3. Scales represent $200\,\mu$. A–D, *D. discoideum*; E, *D. purpureum*. A–C, differences in cell
density and progress of association. A, the centre is at the left. B, the centre is half the width of
the field beyond the left edge; the massive streams at the top are oriented towards the cells
in the bottom half. C, the cells on the left look as if they have been released by the disintegra-
tion of streams; in fact, they have not yet entered streams. D, *1*, the area previously almost
entirely covered by part of a single stippled aggregation, photographed $\frac{1}{2}$-h after returning
to room temperature after 2 days at $2°$ C. Partial reconstruction of the most advanced stream-
lets. *2*, the upper part of *1*, 1 h later. The majority of structures are rings. E, *1*, streamless
centres, the one at the bottom already disintegrating. *2*, $1\frac{1}{2}$ h later. All disintegrated; the sites
of those affected most recently are still recognizable

Timing of the Acrasin Relay

Although a centre cannot attract cells that are more than a fraction of a millimetre away if there are no cells in the intervening space, an aggregation may extend for centimetres: the centre's influence is 'relayed' by the peripheral cells, which when exposed to relatively little acrasin begin to secrete it.

Using a method of estimation devised by Bonner (1949), it has been found that a centre and a stream often secrete acrasin at concentrations that are much the same, judged in terms of the effect on the amoebae (Shaffer, 1957a). By another of Bonner's methods it has been shown that there may be no continuous centrifugal decrement in acrasin emission either along a compact stream or within a stippled aggregation; and indeed it would have been remarkable, in an aggregation of this type, if the organism *had* been able to establish over the whole distance covered by the relay system a nicely graded differential that was not disturbed by considerable local fluctuations in the density and physiological condition of the cells. Now, if all the separate sensitive cells in an area were *simultaneously* induced to begin secreting acrasin at about the same concentration, while they were still unoriented and randomly distributed, each would attract all its neighbours, minor irregularities of distribution would be magnified, and a large number of small clumps would be formed, which would then join up into bigger and bigger aggregates. However, secretion does not in fact start simultaneously throughout an area; and it is to the time and space relationships of the spread of the acrasin relay outwards from the centre that the orderliness and basically radial pattern of aggregation are due.

These relationships depend on a rather complicated set of variables, of which most at the moment are a matter of conjecture. Thus it is possible that if a sensitive unintegrated cell is exposed to an acrasin stimulus adequate to induce its integration, the time lag before it begins to secrete, the course of its secretion, and/or the maximum concentration it produces at its external surface will depend in part on the strength of the stimulus. The precise pattern of stimulation it experiences as it approaches a single source secreting at a constant rate will be determined by the speed at which it orients and advances, which in turn may be affected by the stimulus strength. All the responses of the cell will vary also with its physiological condition, which may change both spontaneously and as a result of exposure to acrasin—that is, it may adapt to the stimulus. And in actual aggregation, instead of a single static source, there may be large numbers of them, distributed irregularly at distances dependent on the average cell density, moving at different and varying speeds, secreting at different and varying rates, and mutually interacting. There is also the question of when, where, and in what concentration the enzyme inactivating acrasin is secreted, of how its secretion is related to the acrasin concentration, and of its subsequent fate. Additionally, if the molecules produced by its extracellular activity are absorbed by the cells, built up into what they were before, and resecreted, or if their structure is suffi-

ently like what it was before for them to compete for the same component
f the cell surface, *their* distribution may be important too. As so little is
nown of these matters at present, no more than a preliminary analysis will
e attempted here of the relationships responsible for the patterning of aggre-
ation in a field of cells that are initially well separated.

The absence of visible local association of the cells in any given field may
e taken as evidence that they are either not secreting enough acrasin to
ffect one another's movements or have only just begun to do so. For if they
ad been secreting for some time, they could have remained separate only
 they all had been moving at the same speed in the same direction, whereas,
 fact, cells advance at different speeds; and, in addition, their being initially
cattered at random, and their being forced ever closer together if they crawl
dially towards a centre, would have enhanced the effect of local attraction.
hus when nothing is to be seen of an aggregation but a single clump, it is
ear that there is no significant secretion by the cells outside it (with the
ossible exception of those moving towards it that are in its immediate
icinity) and no effective propagation by the relay mechanism.

When propagation does occur, streams may develop that from their incep-
on are continuous and grow out rather slowly from the centre. Again, of the
eparate cells, at most those near the tip of a stream can have started to
ecrete, and if they have, they are unlikely to do so maximally till they have
oved still nearer to it. Once in the stream, they can be guided by the flow
f their neighbours, and so it suffices if an adequate gradient is maintained for
 relatively short distance outwards from the tip.

Continuous streams may appear even when the cell density is low, and
ey may in some areas be parallel rather than radial. Their formation is
elped by the elongation of the cells in the direction of the gradient and the
onsequent reduction of the gaps between them, but essentially it depends on
e lateral condensation of comparatively wide bands of cells. Differences in
eed may be involved too: individual cells pursuing the outer end of a stream
ay rapidly catch up with it. (Similarly, a length broken off a stream spon-
neously may follow and later rejoin the central stump.)

When the ratio of the speed of propagation by the acrasin relay to that of
entripetal movement of the separate responding cells is much higher, the
ggregations are initially stippled, their streams discontinuous. The order-
ness of orientation may be explained as follows. As an integrated area invades
 unintegrated one, cells just ahead of its advancing front are attracted
owards it. Even if they soon release acrasin at about the same concentration
 the cells ahead of them, and those behind them do so too, there will still
e a gradient in the same direction as before during the phase in which their
ecretion is increasing, because of differences in the time of onset of secretion.
urther, if by the time all its neighbours are secreting maximally a cell has
urned and extended towards the sources that first attracted it, these will
nd to exert a somewhat greater influence on it than the newer ones, simply
ecause a cell is guided by the external gradient at its *front* end. Whether

this effect is significant or not will depend on the distances separating the cell (as compared with their lengths).

Sometimes the cells in a stippled aggregation reorient towards another centre, which shows that it is possible for the acrasin relay to be re-excited. This suggests that the original centre may be able to re-excite the relay too, and if it can, and does so periodically in those cases where it retains control over the field around it, it is easier to explain the continued movement of the cells towards it. Instead of there being present constantly or recurrently a differential in secretion finely graded from the centre out to the edge of the aggregation, and controlled in a way not easily imagined, there will be an adequate gradient in comparatively narrow zones that travel out from the centre, and the gradient will result simply from time differences in the activation of the sources. Once the cells in a stippled aggregation have joined up into more or less continuous streams, further pulses will be less necessary, though as the cells may be reoriented even at this stage, presumably they may occur.

What is taken as visible evidence for the repeated excitation of the relay system is the rhythmic movement that may be revealed by time-lapse films, especially those of early aggregation (Arndt, 1937; Bonner, 1944); pulses of rapid centripetal movement are propagated centrifugally; and as they are not halted by gaps between the cells, they can hardly be of mechanical origin.

As yet, almost nothing is known quantitatively about cyclical changes in the properties of the cells. The maximum secretion reached in any given excitation of the relay might be maintained and serve as the base level in the succeeding one; but if, as is more probable, there is an interim decline, the gradient will be reversed after each pulse. Some reasons have already been put forward (Shaffer, 1957a) as to why this need not vitiate the orientation mechanism within a stream. While the cells are still separate, there is an additional factor: changes in velocity can alter the distances between them; so that if the zone in which secretion levels off or actually declines coincides with that in which velocity decreases again, the waning of the influence of a given cell on those behind it may to some extent be offset by their closer approach to it.

After stippled aggregations of *D. discoideum* had been cooled to about 2° C. for 1–2 days, their cells had slightly disassociated, though it was possible to recognize the positions that had been occupied by the more compact sections of the developing streams. If the cultures were then returned to room temperature, these sections tended to re-form, though they did so somewhat irregularly and not many of them entered the original centre. The renewed association of the rest of the cells was still more irregular: rings and hemispherical clumps, some of them fed at first by short or rather tortuous streams, appeared in large numbers, perhaps several hundred within the area previously covered by a single aggregation (fig. 3, D). Those that were big enough eventually produced miniature fruiting bodies: but most were too small to do this, and except for those that disintegrated or turned into

ear streams (Shaffer, 1957*b*) and then joined up into bigger organizations,
y persisted long after cells in a neighbouring part of the culture had had
e to feed on bacteria, aggregate, and build fruiting bodies. (However, if
cells were released from their sterile configurations early enough by being
ept together into larger heaps, their development continued.) On return
room temperature, the cells were largely separate, individually polarized,
tually adhesive, and motile in varying degree; their behaviour was con-
tent with their secreting acrasin without any regular differential. I believe
t aggregation ceased to be radial because the temperature changes upset the
nporal sequence of secretion, though just how they acted can only be
rmised. By exposing sensitive unintegrated cells uniformly to a solution
acrasin, it should prove possible to disturb the relay system in a way that
more readily explicable.

The relationships of the relay were not constant within a species. But at
e densities used, in an aggregation of *P. violaceum*, streams usually either
re lacking or slowly grew out from the centre as virtually continuous
uctures; whereas in *D. discoideum* they commonly were discontinuous in
eir early stages and covered much of their ultimate extent almost as soon as
ey became recognizable. All three patterns might be seen in *D. purpureum*.

DISINTEGRATION

In *Polysphondylium violaceum*

If unaggregated cells or those from disintegrated streams were swept
gether with a glass rod into a number of small heaps, these immediately
gan to dissolve, and within the first minute or two the cells had separated
preciably. (In a small proportion of the heaps a tiny knot of cells was left
hind; possibly they had been damaged or had formed an inchoate centre.)
the other hand, if heaps were built of cells obtained from intact streams,
ly some of them dispersed (Shaffer, 1957*a*, *b*). The point to be noted is that
en these did not begin to do so for 5–10 min or more, and then their cells
parated as fast as those from unintegrated heaps. This time-lag suggested
t disintegration, including the loss of adhesiveness, was an active process;
ough possibly the disturbance of the cell surface produced by mechanically
parating the strongly bonded cells was responsible for it.

As to the causation of disintegration, some of its complexity was revealed
the following observations. A stream sometimes disintegrated if the centre
o which it was flowing was removed, but it did not do so invariably. Like-
se, if a stream was cut, integration might be maintained, or disintegration
ght spread upstream from the gap, although this might be no wider than
t over which the cells could originally have received the stimulus to become
tegrated. The spread of disintegration could reasonably be ascribed to a
ain reaction in which, as each cell was affected, the one behind it was left
posed and was thereby stimulated to return to the unintegrated state. This
action might travel along a stream at about an amoeba-length a minute or

much more slowly; and just as the initial cutting of the stream might fail to start disintegration, so at some point the cells might prove resistant to the transmitted stimulus, in which case disintegration was halted. Thus, loss of contact with integrated cells was not a sufficient stimulus. But neither was it a necessary one. In the first place, a stream that had never made contact with the acrasin source that induced it sometimes persisted when it was removed, yet at other times it did not. Secondly, disintegration might start spontaneously in an intact stream, first affecting cells whose front ends were touching integrated cells. If disintegration occurred in other intact streams soon afterwards it was found in adjacent streams more frequently than would have been expected had its position been determined entirely by chance. This being so, it could be assumed that once it had begun in a neighbouring area there was a reciprocal influence on its further progress in the original one.

These results showed that the integrated state was not equally stable in all stream cells. In some of them it was lost following any of a number of visible changes in the aggregation, all of which probably acted by sharply decreasing the acrasin concentration to which they, and especially their front ends, were exposed. In others, which can be said to have stood higher on the 'social scale', it was maintained despite such changes; whereas in those at the low end of the scale, its loss was not preceded by any visible changes, and the cells could have experienced at most a minimal external stimulus—one without effect on their neighbours—and possibly none at all. Additionally, at least a fraction and perhaps all of the stream cells must to some extent have adapted to the integrative stimulus. This adaptation was lost if the cells returned to the single state, for they could then reorient to an acrasin source and integration could be reinduced (with the proper procedure) at a distance from it. The concepts 'variable stability' and 'adaptation' as used here are purely descriptive: for them to acquire an explanatory value they will have to be interpreted in physico-chemical terms.

Disintegration could alter the pattern of cell association in a variety of ways, of which the simplest was the replacement of a minute streamless centre by a small patch where the cells were temporarily rather crowded together (cf. fig. 3, E). In an aggregation with tributary streams, disintegration might appear at one or more points in any or all of them and then spread along them centrifugally, destroying the organization. In some cases it stopped after a short distance; in others it crept through every branch it met right out to the tips of the finest tributaries. If it affected only the outer ends of the streams, the cells involved returned to the condition they were in a short time previously; and the aggregation as a whole still presented a 'typical' appearance—it was merely somewhat smaller. Usually, the separated cells later re-formed streams, continuous with the main system, in the same areas as before.

However, if disintegration spread out from the centre along all the streams, the pattern was radically altered (fig. 4, A). What was left was like a fungal fairy ring, which increases in diameter as the mycelium grows outwards in all directions while dying away in the middle. In a symmetrical aggregation of this
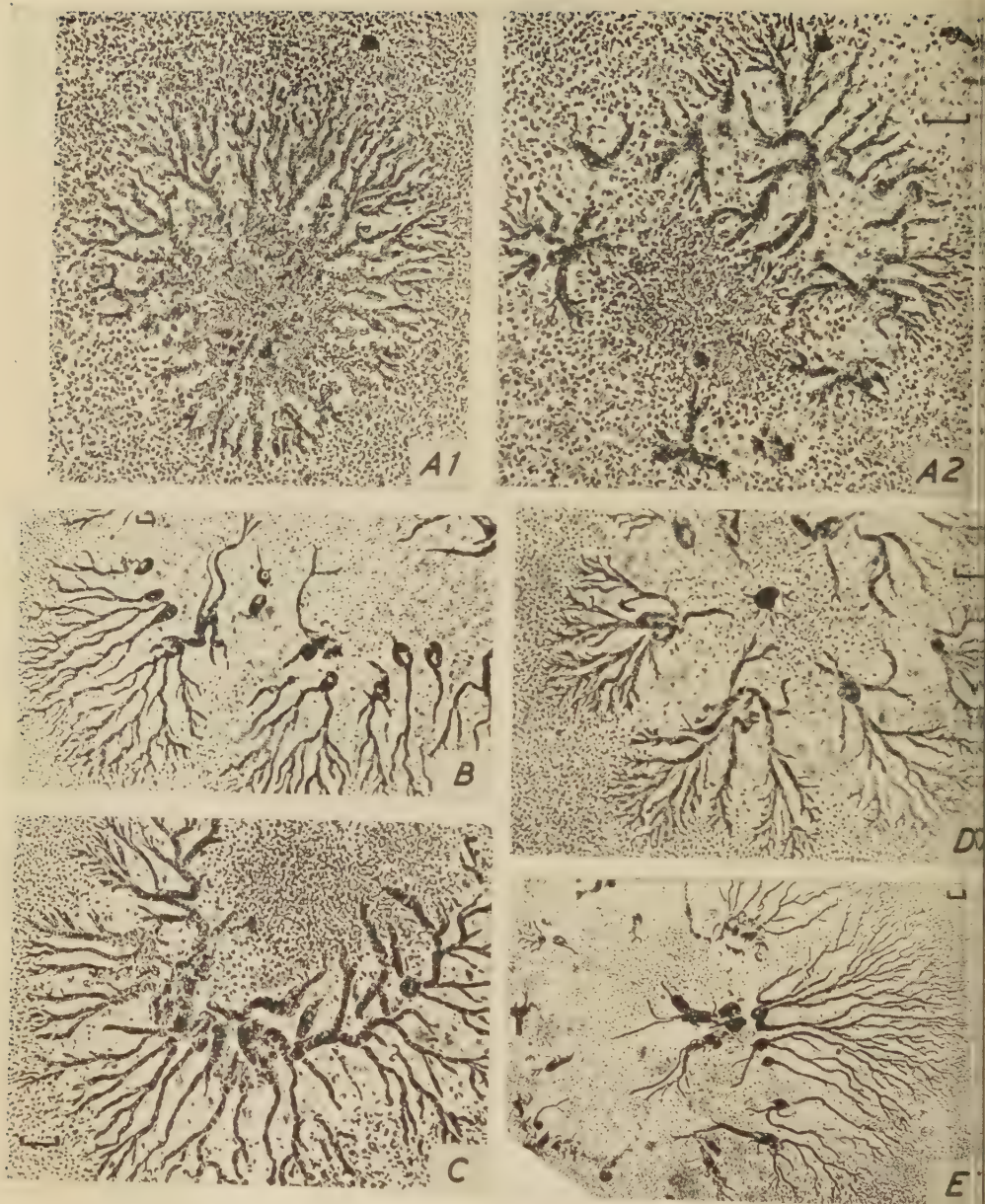
FIG. 4

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ype, the 'ring' was the area of persistent cellular organization; it was bounded
y two concentric circles between which the streams stretched more or less
adially. The outer circle represented the site of incorporation of single cells;
he inner, that of their release. Wide variation in the relative rates of expansion
f the two circles indicated that the two processes were independent. If the
nner circle moved more quickly than the outer, the lengths of the streams
ecreased; in the limiting case, the inner caught up the outer and all organiza-
ion was annihilated. If the inner circle moved more slowly, the streams
engthened. If disintegration stopped, the streams then piled up as solid
masses at their inner ends (fig. 4, A 2) or continued onward, guided by acrasin,
nd so, according to the spacing between them, either looped back on them-
selves (fig. 4, B) or fused more or less extensively with their neighbours (fig.
, C). Whatever their precise course, they attracted the recently separated
cells from the central area. Thus the original aggregation was replaced by a
variable number of discrete organizations, each with streams growing out-
wards made of cells moving inwards, and others growing inwards of cells
moving outwards; this, of course, had not necessitated a reversal of polarity
f any bound integrated cells.

If the original centre had persisted (fig. 4, D) and was secreting acrasin,
t became the nucleus of a new radial aggregation, which was constructed out
f the cells separated from the first one. If this continued to disintegrate, the
new one could grow extensively, but it in its turn might soon start to dissolve.
t was, however, uncommon for one complete 'fairy ring' to form inside
nother, because actual aggregations were almost always asymmetrical: the
streams were not of the same length; they grew into areas of different cell
densities and sensitivities and so extended at different rates; and even if
disintegration began at the centre, it might be arrested at various distances
rom it (fig. 4, E), so that some streams were completely destroyed and others
persisted independently. Yet in limited sectors, there might be half a dozen
concentric bands alternately containing integrated and unintegrated cells.

When, in an approximately radially symmetrical disintegration, the opposed
activities at the inner and outer ends of the streams proceeded at about equal
rates, the streams remained the same length, though the cells of which they
were composed were constantly changing. The streams moved outwards; the
cells inwards. If the cells within a stream had not moved, if they had merely

FIG. 4. *P. violaceum.* Scales represent 200 μ. A, a fairy-ring aggregation. *1*, disintegration has
ust reached the outer ends of the shortest streams. *2*, disintegration has stopped: the streams
ave continued their outward growth (the foreign body at the top is a useful marker) and also
ttracted cells from within the ring. B, part of an aggregation like that in A *2*, but the inner ends
f the streams have looped back on themselves. Most of the de-integrated cells have been
ollected again, but there are still a few reversed streams. C, like A *2*, but the inner ends of the
treams have anastomosed. Disintegration has started again in the streams on the left.
, the original centre has persisted and reattracted some of the de-integrated cells. E, disintegra-
ion, travelling out from the centre, spread in the left half of the aggregation almost or com-
letely to the periphery, but on the right it stopped much sooner. Note reversed streams, and
rebuilt streams flowing into the left side of the original centre

attached themselves at one end of it and freed themselves at the other, they would have been no more crowded inside the 'fairy ring' than outside it. But because they did move they became concentrated inside. The effect was plainly visible (fig. 4, A, C) and persisted, if the ring remained entire, until most of them had been reintegrated in streams; but if some of the streams disintegrated completely, the accumulated cells could leak out through the gap. The cells inside a rapidly expanding ring had the same properties as those outside it: having little intercellular adhesiveness, they were separate and independent, and they were motile but undirected. A ring of streams thus transferred cells of a particular type from one concentration to a higher one; it acted by imposing a uniform orientation on its polarized cellular components and disciplining their inherent motility. It was interesting that though this polar organization was called into existence by a centre, it did not need one for survival. It was not based on unchanging morphological elements: it was dynamic and self-perpetuating; it depended on a 'tradition' of polarity being handed on to the entering cells, which if it was once lost could not be re-established without the intervention of an acrasin source.

(ii) In *Dictyostelium discoideum*

Disintegration as it occurred in *D. discoideum* was much the same as in *P. violaceum*, the major differences being in the residual adhesiveness of the de-integrated cells, as described already (Shaffer, 1957a), and in its timing: when compared with the speed at which the cells moved, the spread not only of integration but of disintegration too tended to be more rapid in *D. discoideum* than in *P. violaceum*; so that instead of the cells in a stream detaching themselves in an obvious sequence, visible dissolution might start almost simultaneously throughout the whole of the length affected or at least a considerable part of it. Time-lapse photography showed also that the output of acrasin fell abruptly at the same time, as far as could be judged by watching the disorientation of single cells that were being attracted. After multiple sectioning of a single stream or the removal of the centre from an aggregation, a number of the fragments might disintegrate perhaps 5, 10, or 20 min later. Some or even all of them might begin to disperse at almost the same moment or at widely different ones, and a large fragment might break down in several discrete steps.

Much remains to be determined of the causation of disintegration and of the role taken by changes in the acrasin field.

Onset of Aggregation

It has been generally agreed that aggregation does not usually occur till almost all the 'available' food has been eaten. Potts (1902) cultivated the amoebae in the vegetative phase indefinitely by transferring them periodically to fresh supplies of bacteria. Raper (1940b) carefully covered aggregations with a suspension of bacteria and found that only when the cells were disturbed did they return to the solitary state and feed on them. Arndt (1937) reported that

ome time after a plate of edible bacteria had been inoculated with spores of
. *mucoroides* at one point, concentric zones were occupied by: fruiting
odies, aggregations, the preplasmodium (a loose association of preaggregation
ells), and feeding cells; this showed that the social phase began when the cells
xperienced a certain degree of starvation. However, in very sparse cultures
ggregation was delayed several days, while the cells formed small groups by
andom collision; but if they were gathered together earlier, they at once
eveloped as true aggregations. Cell contact was thus an additional factor.
Vorking with *D. discoideum*, Raper (1940*b*) found aggregation to occur sooner
a thin cultures on media poor in nutrient than in luxuriant ones on rich
aedia and concluded that contact was not a controlling factor; it was possible,
hough, that it was one, but that it acted only after the cells were without food.
aper reported that aggregation began about 42–44 h after nutrient-poor
gar had been uniformly inoculated with bacteria and *discoideum* spores.
f the cultures had been grown in daylight instead of darkness, or if, 6 h before
ggregation would otherwise have been expected to occur, they had been
aildly dried or had had their temperature slightly raised, aggregation started
few hours sooner. Also, under these conditions, the aggregations were smaller
ad more numerous; but perhaps this was simply because centres had
ecome active more nearly at the same time.

If degree of starvation and possibly cell contact were the main factors
avolved, a group of replicate cultures started every few hours with amoebae
r spores evenly distributed through the bacteria should contain aggregations
aroughout the day; so should a group of cultures started simultaneously with
ifferent amounts of bacteria. In the present work, this was found not to be
ae case in *D. discoideum*: there was a strong tendency for new aggregations
o appear in outbursts lasting a few hours and rather well synchronized in
ifferent cultures, even in those showing between them a wide range of popu-
tion densities. A plate of bacteria developed concentric zones of fruiting
odies and migrating aggregates immediately around the point where *dis-
oideum* spores or amoebae were inoculated, and an expanse of feeding cells
arther out. But the area in between did not contain all the intervening stages
f development: for periods of perhaps 12 h or more, there was nothing it in
ut preaggregation cells; then, in many cases within 2–3 h nearly all of these
ere marshalled into aggregations, which transformed into migrating aggre-
ates about 8 h later. Beyond them widened a zone of cells that had stopped
eeding and were awaiting the next burst of social behaviour.

In contrast, with a strain of *D. mucoroides* that formed a preplasmodium,
oint-inoculated cultures showed almost continuously all the zones that
rndt had described; and replicated spread-cultures started at intervals
ggregated in succession at about the same intervals. This difference in
ehaviour from *D. discoideum* was maintained even when the two species were
rown on alternate agar sectors inside the same Petri-dish and were thus
xposed to almost the same environmental stimuli.

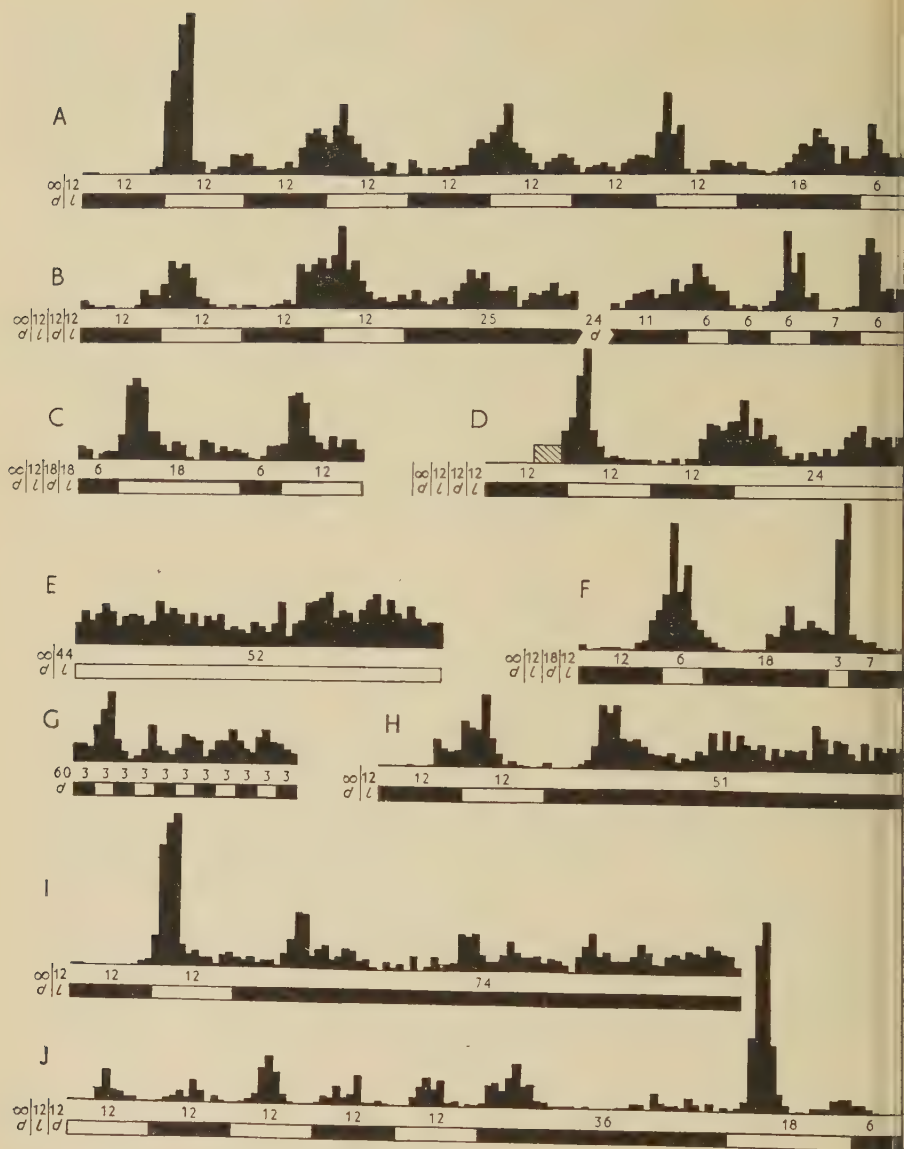In an attempt to determine the factor that stimulated *D. discoideum*, cultures

Fɪɢ. 5. Hourly counts of apparently primary centres. The culture plates were spread with
bacteria uniformly and inoculated with *D. discoideum* spores along one-third of the circum-
ference. Periods of light and darkness are indicated by the strips under the histograms;
figures are hours. The earlier illumination of the cultures is shown on the left of the strips:
l, light; *d*, darkness; ∞*d*, darkness continuous from inoculation. All the histograms are to the
same scale, the highest peak in A representing 120 centres. No account is taken of the ultimate
size of the centres or of variation in the area of bacteria consumed per h. Cultures were kept
in constant temperature rooms: A–I at 26° C, J at 16° C. A, the outburst at dawn is split into
two components by lengthening the period of darkness from 12 to 18 h. J, the two components
are well separated even when the cycle is 12-h darkness, 12-h light. The shaded area in D
represents the centres formed during the dark period as estimated at the subsequent dawn.

f this species, inoculated in a limited area, were variously exposed (fig. 5) o light and darkness, and hourly counts were made of the new aggregations hat appeared (using, in the dark phase, momentary illumination by weak ed light). After each change from darkness to light, or 'dawn', there was an utburst of aggregation. In one experiment, with cultures kept at 26° C, this egan in the first hour, reached its peak in the third or fourth hour, and then eclined; at 16° C the peak came 2 h later. At 26° C, bursts followed each lawn when there were alternating 6-h or even 3-h periods of light and dark- ness. With 12-h periods they apparently anticipated the second and sub- equent dawns, beginning a few hours before them. Investigation showed that his was due to the fusion of outbursts in pairs: one induced by light with nother that at this particular temperature developed in the dark about 20 h fter the previous dawn. An outburst of the second type occurred alone when . culture was placed in continuous darkness after at least one dawn, and it vas followed at intervals by other bursts that became progressively less dis- inct. The probable explanation was that the aggregation stimulated by a ingle period of light collected all the cells that might otherwise have initiated :entres during the ensuing hours, and that there was then a considerable lelay before a further crop of cells had developed, in the dark, to the point vhere they could start aggregation. This again removed any cells on the hreshold of social behaviour, and so the process continued; but as the clear- ances of a culture were slower and less thorough in darkness, synchrony leteriorated with each burst.

A culture in which the cells were widely separated and one in which they ormed a continuous sheet tended to yield simultaneous outbursts when acti- rated by light.

It is not clear that all known cases of synchrony can yet be accounted for :ven in *D. discoideum*, and in *D. purpureum* and *P. violaceum* the appearance of lew aggregations followed a more complicated pattern. Moreover, especially n these species, it sometimes happened that a large proportion and even a najority of the centres spontaneously disintegrated within an interval as hort as an hour (fig. 3, E); possibly chemical influences were involved.

THE INITIATORS

The initiation of a primary centre is a process that is still little understood but is clearly rather complex. For example, unaggregated, acrasin-sensitive :ells of *P. violaceum* could be piled up into heaps after numbers of minute treamless centres had formed spontaneously in the population; yet even if the leaps were much larger, they did not usually themselves develop into new :entres, but dispersed. Later, when the cells were at a considerably lower lensity and had much less contact with one another, the same population night produce further centres; but again, it might not do so.

Quantitative studies on initiation were hindered by the difficulty of deter- nining just how many primary centres had formed in a culture. Even at the ame population density, aggregations might show a considerable size range

which varied with the rate at which primary centres developed. Spontaneous breakage and disintegration led the larger aggregations to increase in number by fragmentation, and the smaller ones to disappear without trace. In addition, the cells released by disintegration might form further primary centres, and in some species, unaggregated and de-integrated cells might temporarily or permanently lose their acrasin sensitivity.

Sussman and Noel (1952) found that, at population densities that they claimed did not limit centre formation, the number of centres they counted was proportional to the number of amoebae present, there being, for example, 1 per 2,110 cells in *D. discoideum*. As the count at first increased with the population density and then fell off again rather rapidly, there may in fact have been a significant limitation when the maximal number appeared, because this peak could have occurred when some density-dependent inhibitory process outstripped another density-dependent process increasing the number of centres. Thus, even if their method enabled them to make an accurate count of the primary centres, the precise significance of their figures was doubtful.

These authors also examined small, replicate population samples and found that not all of them produced centres; they considered this to be evidence that by the end of the growth period the population was heterogeneous with respect to the ability to initiate aggregation. Perhaps, though, all that could safely be concluded from their data was that initiation involved a change in the cells that occurred with a certain low probability in the conditions used.

Primary centres are founded by cells that become integrated when there are no other integrated cells near them, and they may gain (though not necessarily retain) control of the movements of the surrounding cells simply by being the first to set up acrasin gradients adequate for orientation over even short distances and the first to activate the acrasin relay. However, it has proved extremely difficult to decide whether the initiator of a centre may be a single cell or whether it must be a small group. Direct visual observation tends to detect a centre too late to be sure, and the time-lapse films so far made that record the development of a centre have been taken at too low a magnification or too high a cell density to resolve this point. Besides, if orientation did seem to be initially towards a single cell, this one might just be the only stationary member of a group of acrasin secretors. Even if it were at that moment the sole secretor, some contact between cells might have been essential at a slightly earlier stage.

Sussman (1952) attempted to settle the question by examining the formation of centres in mixed populations of the wild type of *D. discoideum* and a mutant of it that was unable to aggregate when by itself but was attracted to wild-type centres. When the density of the mutant cells was increased, the number of centres formed in the presence of a given number of wild-type cells rose, but it eventually reached a maximum value. As this maximum varied directly, and not exponentially, with the number (and therefore the density) of the wild type, it was concluded that no collaboration between wild-type cells was necessary and that the initiators were single cells. This argument

ested on the assumption that the mutant was unable to contribute to the production of the aggregative stimulus. However, the evidence published was that when the wild type was dispersed to such an extent that, alone, it could not aggregate, the addition of mutant cells permitted the development of centres and normal fruiting bodies, in which only wild-type spores were detectable. The two cell-types must therefore have been able to promote *each other's* aggregation; and until the nature of the interaction is known (perhaps there was a transfer of acrasin precursors), the possibility that the initiators contained mutant cells cannot be excluded. (The cells of this mutant placed on a thin agar membrane merely collected opposite wild-type centres that were on the other side and did not form streams of their own (Sussman and Lee, 1955); and one presumes this was because, by themselves, they were unable to operate the acrasin relay. The same agar barrier frustrated the synergistic development of all the pairs of morphogenetically deficient strains that in mixed populations could develop further than could either partner separately (Sussman, 1954).) Furthermore, the wild-type cells were never present in the mixtures at less than a tenth of the density at which they produced the maximum number of centres when by themselves. Were most of them needed only to maintain established centres, or did initiation require some form of interaction between wild-type amoebae, as well as between wild type and mutant? If their density influenced only some steps in the sequence leading to initiation, it may well not have been limiting in the conditions used.

When cells of the mutant *fruity* were mixed with the wild type, centres formed in excess of the number that would have been produced by the wild type alone; and Sussman (1955*b*) concluded that these were each initiated by a single mutant cell. Here again, his own evidence—that *washings* of *fruity* cells increased the number of centres formed by pure wild-type populations—vitiated his conclusion.

The maximum number of centres built by the unmixed wild type was 1 per 2,110 cells, but more appeared in the presence of a very high density of *aggregateless* cells, the precise increase depending on the particular *aggregateless* stock used. Sussman (1952) tentatively supposed that there were differences in initiating capacity in the wild type, and that the number of centres actually formed depended on the density of the 'responder' cells and on their sensitivity to the initiator's stimulus. However, on this basis it is hard to explain why no more initiators were revealed in high-density wild-type populations when the possibility of interference between initiators was excluded (Sussman and Noel, 1952; this result was obtained when these authors were still seeking evidence that the initiators were single cells, and was cited as such). In any case, the method of analysis used could not in reality distinguish between a response *to* the initiators and a response *of* the initiators. The so-called 'responder' cells may, of course, have participated at more than one stage, before, during, or after initiation. (If they did so, the presence in a mixture of wild type and *aggregateless* of a second *aggregateless* stock *less*

'sensitive' than the first might well, with some pairs of mutants, have yielded additional centres.)

Recently, Sussman (1956) has presented evidence that the initiators arise not via a stable genetic alteration but by the exploitation of random physio-logical differences; however, it has still to be determined at just what stage the relevant biochemical heterogeneity appears in the population, whether there is any corresponding visible heterogeneity, and whether a single cell can consummate the initiation process. One can scarcely hope for a real understanding of initiation till it has been described in biochemical terms. For the present, one may tentatively suppose: firstly, that the initiators are distinguished by their ability to release acrasin into the surrounding medium without there being any there already or without their being sufficient to induce the remaining cells to secrete; secondly, that this ability is either the immediate or the indirect consequence of the chance appearance of a peculiar and rather improbable configuration within amoebae that are temporarily in a particular physiological condition; and thirdly, that the environment pro-vided by the rest of the population exerts a considerable influence.

SEXUALITY

The existence of sexuality in cellular slime moulds was first claimed by Skupienski (1918); he was able to observe syngamy occurring though he found it to be inhibited by the least light. On his findings then, syngamy could not be an essential part of aggregation, for this readily takes place in daylight. Wilson (1952, 1953) believed aggregation to be basically a sexual phenomenon: the centres were started by the first zygotes to form, and as others appeared they joined the streams; meioses took place during aggregation but were not synchronized. His evidence was cytological and taken from stained culture smears; these were doubly inadequate in that they could show neither the real sequence of development, as a study of the living cells could have done, nor even the actual positions of the fusing and dividing cells in the organization, as would have been revealed by cultures fixed and stained *in situ*. Many objections to Wilson's interpretation are discussed in Sussman's review (1955a), though that based on the quantitative study of initiation is not as serious as he supposed: mention will be made here only of findings in the present work that are not compatible with the view that aggregation is essentially based on syngamy.

1. Visual observation shows that cells may enter streams without first forming zygotes and, in thin streams where observation may be easily con-tinued, may not form them later.

2. Single sensitive cells can be attracted to a natural or an artificial acrasin source and can also be induced to make acrasin. It is therefore hard to see what vital role syngamy could take in the growth of an aggregate.

3. The same cells may undergo integration and disintegration several times. If these changes were associated with a cycle of fusion and meiosis, one might expect there to be a noticeable reduction in cell size: this has not been observed.

4. Amoebae are able to invade and eat a layer of bacteria sandwiched between agar and a coverslip and then aggregate in this position. If a fixative is added, they adhere to the glass. So it is possible to obtain preparations, stained with aceto-orcein, containing feeding cells, preaggregation cells, and entire and largely monolayer aggregations, in their natural positions. Every nucleus in all the streams and in the separate cells just beyond their outer ends may be in interphase.

The possibility that syngamy does sometimes occur cannot of course be excluded. However, as the genetic make-up of the fruiting body depends on that of all its component cells, there may still be some 'recombination' even in the absence of a sexual cycle, if there is any naturally occurring, genetically determined variation in those properties of individual cells of importance in fruiting. Selection will favour fruiting bodies with a genetic constitution that best adapts them to the microenvironment.

REFERENCES

ARNDT, A., 1937. Roux' Arch. EntwMech. Organ., **136**, 681.
BONNER, J. T., 1944. *Fruiting in Dictyostelium.* Harvard Univ. (A film.)
—— 1947. J. Exp. Zool., **106**, 1.
—— 1949. Ibid. **110**, 259.
POTTS, G., 1902. Flora (Jena), **91**, 291.
RAPER, K. B., 1940a. Amer. J. Bot., **27**, 436.
—— 1940b. J. Elisha Mitchell Sci. Soc., **56**, 241.
—— 1941. Growth (Suppl.), **5**, 41.
SHAFFER, B. M., 1953. Nature, **171**, 975.
—— 1956. J. exp. Biol., **33**, 645.
—— 1957a. Quart. J. micr. Sci., **98**, 377.
—— 1957b. Ibid. **98**, 393.
—— 1957c. Amer. Nat. (In press.)
SKUPIENSKI, F. X., 1918. C.r. Acad. Sci. Paris, **167**, 960.
SUSSMAN, M., 1952. Biol. Bull., **103**, 259.
—— 1954. J. gen. Microbiol., **10**, 110.
—— 1955a. Biochem. and physiol. of protozoa, vol. 2, p. 201 (Hutner and Lwoff, Eds., New York, Academic Press).
—— 1955b. J. gen. Microbiol., **13**, 295.
—— 1956. Biol. Bull., **110**, 91.
—— and LEE, F., 1955. Proc. Natl. Acad. Sci. Wash., **41**, 70.
—— and NOEL, E. 1952. Biol. Bull., **103**, 259.
WILSON, C. M., 1952. Proc. Nat. Acad. Sci. Wash., **38**, 659.
—— 1953. Amer. J. Bot., **40**, 714.

The Origin of the Metazoa and the Stigma of the Phytoflagellates

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SUMMARY

It is well known that in various phytoflagellates one of the flagella is connected with the eye-spot or stigma. In the case of *Chromulina psammobia* the more differentiated structure of the eye-spot + flagellum complex seems analogous to that of a retinal rod with its connective cilium binding the external segment and the cell-body.

It is tempting to draw a parallel between this finding of comparative cytology and a hypothesis on the origin of Metazoa proposed first by J. R. Baker, and subsequently by A. C. Hardy.

ON THE ORIGIN OF THE METAZOA

IT seems reasonable to suppose that in the course of biological evolution, the unicellular organisms, or Protista, preceded the multicellular plants and animals. In such a case, the development of multicellular organization from the unicellular state would imply:

(*a*) that the cells which make up the lineage of a protist have remained united during the course of their successive fissions;

(*b*) that of these cells, some were able to differentiate with respect to the others—as much structurally as functionally—their ensemble constituting a new, organized system endowed with a higher degree of complexity.

In accord with these suppositions we know that the lower plants offer broad potentialities for the transition from the unicellular organization. Thus, a number of phytoflagellates are able to aggregate temporarily in palmelloid form; moreover, multicellularity manifests itself in each of their groups by colonial or coenobial structures, which are achieved in many species before they become really established in the true Algae of the corresponding type.

The case of the Zooflagellata is opposed to that of the Phytoflagellata, as Baker (1948) has pointed out, since the former group shows few indications of the possible origins of the Metazoa. Among the Protozoa, only the Choano-flagellata can, through their aggregates, lead to a group of Metazoa—but to a very specialized group, the sponges; and if one examines the case of the Cnidosporidea with their pluricellular, differentiated states, the question arises as to whether they actually do represent Protozoa, and not Metazoa extremely degenerated by parasitism (see Poisson, 1953).

Baker believes that the attainment of a multicellular state is, to a large extent, conditioned by the particular mode of nutrition of the aggregated cells. Among the protophytes, exchanges take place across the whole surface

of the body, and this process is not greatly modified when several cells are closely joined to each other. It is otherwise for the Protozoa furnished with a buccal differentiation serving for the ingestion of food, since the close association of such cells would presume that a new and collective manner of nutrition had been realized. Baker concludes from this that only the very primitive Protozoa lacking localized, definite alimentary organs could unite to give rise to the first Metazoa. In fact, the most highly differentiated Protozoa are able to form colonies; but their high degree of organization precludes the integration of the different zooids, which accept only a relative interdependence; take, for example, the colonies of heteromorphic *Zoothamnium*, or the remarkable planktonic ciliate, *Ctenoctophrys chattoni*, described by R. Weill (1946) (see Fauré-Fremiet, 1954).

Hardy (1953) has re-examined Baker's arguments, but, carrying the deductions to the end and leaving aside the case of the Choanoflagellata and the sponges, he infers protophytic origin for the Metazoa. Collective phagotrophic nutrition would have established itself among very simple metaphytes after the loss of their chromoplasts; and we know that the evolution of the phytoflagellates offers, without any doubt, rather numerous examples of such an accident, leading to a change of nutrition in the holozoic direction (see Fritsch, 1935; Pascher, 1942).

These hypothetical considerations form a preface to the following remarks.

The Stigma of the Phytoflagellata

Among a large number of phytoflagellates there is an organelle in the form of a granule or a platelet, which is coloured orange-red by a pigment of the carotene type (Wolken, 1956b); this is the 'stigma' or 'eye-spot', considered to be a sensory organelle and, in particular, a photoreceptor (Franzé, 1893, 1908; Rothert, 1914; Mast, 1927, 1938; Wolken, 1956 a, b, &c.). Rothert (1914) showed that the stigma of a phytoflagellate is a derivative of the chromoplasts; Mangenot (1920), Chadefaud (1931, 1935), and Hollande (1938b) have verified this fact by demonstrating precisely that the eye-spot can be a delimited part, a localized differentiation of a plast, as with the Chlorophyceae or the Xanthophyceae for example; or else, as with the Euglenidae, a small chloroplast entirely transformed into a stigma.

The electron microscope has revealed a certain structural complexity of the stigma. In *Euglena gracilis* (Wolken and Palade, 1952, 1953; Wolken, 1956 a, b), *Chlamydomonas reinhardi* (Sager and Palade, 1954, 1957), the spermatozoid of *Fucus* (Manton and Clarke, 1956), *Chromulina psammobia* (Fauré-Fremiet and Rouiller, 1957), &c., the eye-spot is formed by the regular juxtaposition, underneath the membrane of the chromoplast, of a series of elements. These are spheroid, oval, or cylindrical 'chambers' measuring from 100 to 430 mμ. These 'chambers' are limited by double membranes comparable to those which subdivide the plastid; they contain a pigment of the carotene group.

Another remarkable characteristic of the stigma is shown in its connexions with a flagellum. One recognizes, among the Euglenidae, the presence of a

paraflagellar body located in the vicinity of the stigma and presumed to be a photoreceptor; the electron microscope permitted the establishment of this fact (Wolken and Palade, 1952, 1953) and the demonstration of other examples of a flagello-stigmatic coaptation.
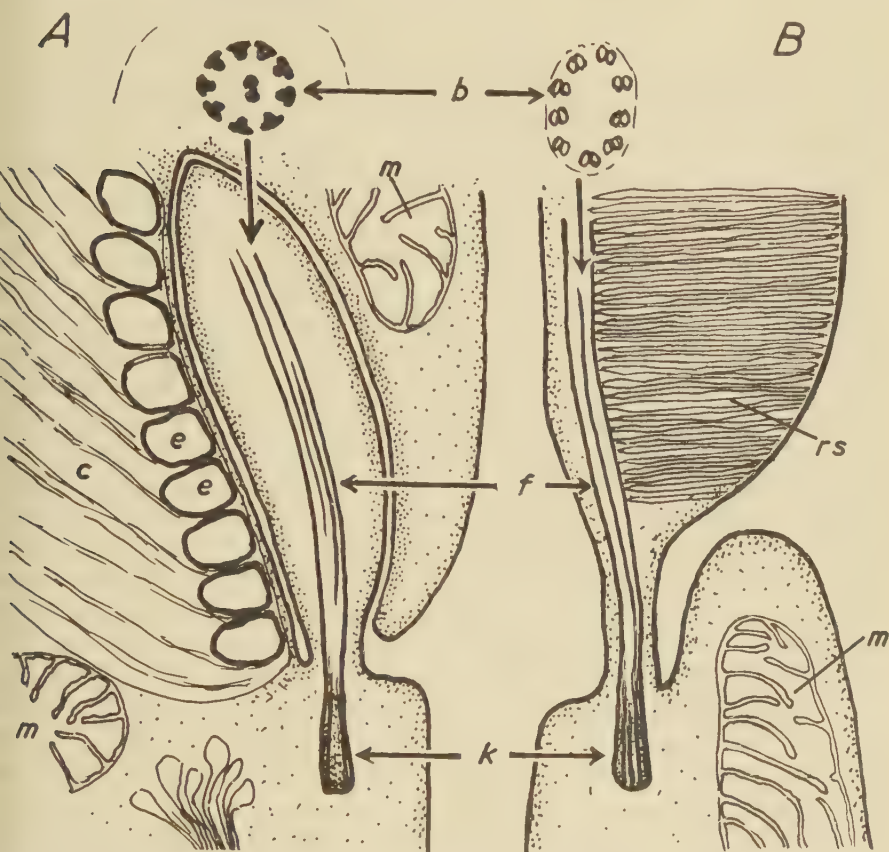

FIG. 1. Composite diagrams based on several electron micrographs. A, eye-spot of *Chromulina psammobia*, according to Fauré-Fremiet and Rouiller (1957). B, junction of outer and inner segments of a retinal rod cell, redrawn after de Robertis (1957). *b*, transverse sections of a fibrillar bundle of *Chromulina* (on the left) and of a retinal rod cell (on the right) (these sections are at a higher magnification than the rest of the diagram); *c*, chromoplast of *Chromulina*; *e*, eye-spot chambers of *Chromulina*; *f*, fibrillar bundle; *k*, kinetosomes; *m*, mitochondria; *rs*, flattened rod sacs of the retinal cell (both the eye-spot chambers and the rod sacs contain carotene pigment)

Thus, in the spermatozoid of *Fucus* (Manton and Clarke, 1956), the recurrent flagellum turns back and touches the surface of the body at the level of the eye-spot; it becomes flat and adheres closely to the cell-membrane along the whole width of the eye-spot, while beyond it becomes free again and cylindrical in shape.

In *C. psammobia* (Fauré-Fremiet and Rouiller, 1957), next to the external flagellum there is a very short internal flagellum, not visible in the living

organism, because it is developed in an invagination of the cell-membrane. It displays the classical structure of a ciliary organelle, with a tubular basal granule and a sheath of 9 double, peripheral fibrils surrounding a double central fibril; but this sheath is surrounded by an appreciable mass of amorphous matrix. This curious internal flagellum is tightly lodged in a depression of the membrane of the chromoplast, in contact with the stigma (fig. 1, A), thus realizing a coaptation comparable to the one already known in the spermatozoid of *Fucus*, in comparison with which the flagello-stigmatic complex of *Chromulina* seems a highly differentiated organelle.

The knowledge of such a complex photo-receptive apparatus in a phytoflagellate suggests a comparison with the retinal cells of the vertebrates.

The External Segment of the Retinal Cells of Vertebrates

Long ago the cytological techniques utilized by Fürst (1904) and Leboucq (1909) showed that a ciliary organelle participates in the morphogenesis of the external segment of the retinal rods and cones; and the studies pursued by Schmidt (1935, 1937) on some of the physical properties of the visual cells, indicated that this same external segment is formed by the layering of lipoprotein lamellae to which the pigment carotene is bound. We are indebted to the electron microscope, through the recent works of Sjöstrand (1953, 1956) and of Robertis (1956a, b) on mammals, and of N. Carasso (unpublished) on the toad, for additional information which is especially important.

The external and internal segments of a rod, for example, are bound together by a fibrillar bundle surrounded by a membrane and corresponding to a cilium, of which the basal granule is enclosed in the internal segment. This is the 'connective cilium' of which the double axial fibril is missing, whereas the 9 double peripheral fibrils cross the intersegmentary neck and spread out along the side of the proximal part of the external segment, in contact with its stack of bilamellar elements, or flattened sacculi, containing the red pigment (fig. 1, B).

The validity of a comparison between this structure and that of the flagellostigmatic complex of *Chromulina* is evident, as Wilmer (1955), on the evidence of some less significant data, has already pointed out. In one case as in the other, the photosensitive pigment, carotene, is distributed through a series of compartments—vesicles or flat sacculi—to the length of which there is closely applied a cilium, whose basal granule is situated farther on, within the cytoplasm. Let us, however, point out some differences:

(a) In the phytoflagellates the fibrillar infrastructure of the cilium is complete, while in the vertebrates it is reduced to the external bundle.

(b) In the phytoflagellates there is a close coaptation of the two distinct organelles: the flagellum, on the one hand, and on the other, the 'carotenophore' or pigmented structure (stigma) differentiated within a plastid; whereas in the vertebrates the pigmented structure is differentiated, according to Robertis, from the 'primitive cilium' at the expense of a 'morphogenic material' contained in its matrix.

These differences do not diminish the value of the suggestions formulated y Wilmer and apropos of which Wolken (1956a) added, concerning the case f *Euglena*: 'We can look upon the *eye-spot + flagellum* as really a "primitive ye" or the most "elementary nervous system".'

THE CASE OF THE PROTOZOA

The Protozoa, lacking chromoplasts, do not seem to have developed the quivalent of the eye-spot of the Protophyta. This fact does not exclude the ossibility of a certain sensitivity to light.

Among the most highly organized Protozoa, such as the ciliates, a certain umber of species are positively or negatively phototactic: *Stentor coeruleus*, rovided with a diffuse, blue pigment, is photophobic; *S. niger*, provided with very peculiar black pigment (Barbier, Fauré-Fremiet, and Lederer, 1956), is hototropic; *Chlamydodon mnemosyne*, a psammic photophobic species, bears diffuse spot composed of an orange-yellow, liposoluble pigment. We know f still other species possessing a pigmented spot without definite structure; he physiological role of the spot has not been determined.

A much more curious case is that of *Strombidium oculatum* Gruber: this narine ciliate lives in symbiosis with a *Chlamydomonadina* (Fauré-Fremiet, 948) which multiplies more rapidly than its host; the latter digests the excess f that population, but the chlamydomonadian stigmata are conserved and ccumulate in the apical part of the ciliate body, forming the 'eye-spot' well escribed by Gruber. Now, it is remarkable to verify that this ciliate manifests very marked positive phototropism, during the entire active phase of its xistence at least. This fact confirms that Protozoa such as the ciliates are nable, by themselves, to realize the differentiation of a photoreceptive or-anelle.

QUESTIONS

The facts and considerations examined in the preceding pages do not justify he formulation of a conclusion; they permit us, however, to trace a parallel etween an hypothesis on evolution and a fact of comparative cytology.

The hypothesis, advanced by Hardy, upsets a current opinion by assuming hat the Metazoa more probably evolved from the Protophyta than from the Protozoa.

The fact is that the external segment of a visual cell of the vertebrates and he eye-spot of several phytoflagellates are comparable in the sense that they oth realize the close coaptation of a ciliary organelle and a vesicular system ontaining the carotene.

The parallel is obviously suggestive, but, before admitting that the fact can upport the hypothesis, one would have to know if it reflects something other han a fortuitous resemblance or some superficial analogy. And if these cilio-arotenophore complexes that had developed in the chrysomonad cell and in he retinal cell could be considered as physiologically and morphologically omologous, other questions would arise at once. One would have to know if

such a cellular differentiation, presumed to be photoreceptive, could have
developed several times in independent ways and by diverse mechanisms, or
if it implies some causal relationship; if the idea of 'plast' is necessarily tied to
that of a carotene-bearing organelle, or if other cytoplasmic structures, of the
Golgi type, for example, could play the same role. Those are the problems of
comparative cytology which the electron microscope will perhaps permit us
to resolve.

REFERENCES

BAKER, J. R., 1948. 'The status of the Protozoa'. Nature, **161**, 548 and 587.

BARBIER, M., FAURÉ-FREMIET, E., and LEDERER, E., 1956. 'Sur les pigments du cilié *Stentor niger*.' C.r. Acad. Sc., **242**, 2182.

CHADEFAUD, M., 1931. 'Sur la signification morphologique du stigma des zoospores et des zoogamètes chez les Hétérokontes et les Phéophycées.' Ibid., **193**, 1030.

—— 1935. 'Le Cytoplasme des algues vertes et des algues brunes, ses éléments figurés et ses inclusions.' Revue algologique, **8**, 1.

FAURÉ-FREMIET, E., 1948. 'Le Rythme de marée du *Strombidium oculatum* Gruber.' Bull. Biol. France-Belgique, **82**, 1.

—— 1954. 'Les Problèmes de la différenciation chez les protistes.' Bull. Soc. Zool. France, **79**, 311–29.

—— and ROUILLER, CH., 1957. 'Le Flagelle interne d'une Chrysomonadale : *Chromulina psammobia*.' C.r. Acad. Sc., **244**, 2655.

FRANZÉ, R., 1893. 'Zur Morphologie und Physiologie der Stigma der Mastigophoren.' Z. wiss. Zool., **56**, 138.

—— 1908. *Die Lichtsinnesorgane der Algen.* Stuttgart (Cosmos).

FRITSCH, F., 1935. *The structure and reproduction of Algae.* Cambridge (Univ. Press).

FÜRST, C., 1904. 'Zur Kenntnis der Histogenese und der Wachstums der Retina.' Lund's Univers. Årsskrift, **40**.

HARDY, A. C., 1953. 'On the origin of the Metazoa.' Quart. J. micr. Sci., **94**, 441.

HOLLANDE, A., 1938a. 'Les Dictyosomes des Eugléniens.' C.r. Soc. Biol., **127**, 517.

—— 1938b. 'L'Appareil parabasal des Cryptomonadines.' Ibid., **206**, 370.

—— 1952. 'Classe des Chrysomonadines' in P. P. GRASSÉ, *Traité de Zoologie*, **1**, fasc. 1, 471. Paris (Masson).

—— 1952. 'Ordre des Choanoflagellés.' Ibid., **1**, fasc. 1, 579.

LEBOUCQ, G., 1909. 'Contribution à l'étude de l'histogenèse de la rétine chez les Mammifères.' Arch. Anat. micr., **10**, 555.

MANGENOT, G., 1920. 'À propos de la signification du stigma des Euglènes.' C.r. Soc. Biol., **94**, 577.

MANTON, I., and CLARKE, B., 1956. 'Observations with the electron microscope on the internal structure of the spermatozoid of *Fucus*.' J. exp. Bot., **7**, 416.

MAST, S. O., 1927. 'Structure and function of the eyespot in unicellular and colonial organisms.' Arch. f. Protistenk., **60**, 197.

—— 1938. 'Factors involved in the process of orientation of lower organisms in light.' Biol. Rev., **13**, 186.

PASCHER, A., 1943. 'Zur Klärung einiger gefärbter und farbloser Flagellaten und ihrer Einrichtungen zur Aufnahme animalischer Nährung.' Arch. f. Protistenk., **96**, 75.

POISSON, R., 1953. 'Sous-embranchement des Cnidosporidies' in P. P. GRASSÉ, *Traité de Zoologie*, **1**, fasc. 2, 1006. Paris (Masson).

ROBERTIS, E. DE, 1956a. 'Morphogenesis of the retinal rods. An electron microscope study.' J. biophys. biochem. Cytol., **2**, 4 (Suppl.), 209.

—— 1956b. 'Electron microscope observations on the submicroscopic organization of the retinal rods.' Ibid., **2**, 319.

ROTHERT, W., 1914. 'Die Augenflecke des Algen und Flagellaten ein Chromoplast.' Ber. deut. Bot. ges., **32**, 91.

ROUILLER, CH., and FAURÉ-FREMIET, E., 1957. 'Structure fine d'un Flagellé chrysomonadien : *Chromulina psammobia*.' Exp. Cell Res. (in the press).

SAEDELEER, H. DE, 1929. 'Recherches sur les choanocytes; l'origine des Spongiaires.' Ann. Soc. Roy. Zool. Belg., **50**, 17.

GER, R., and PALADE, G. E., 1954. 'Chloroplast structure in green and yellow of *Chlamydomonas.*' Exp. Cell Res., **7,** 584.
—— 1957. 'Structure and development of the chloroplast in *Chlamydomonas.* I. The normal green cell.' J. biophys. and biochem. Cytol., **3,** 463.
CHMIDT, W. J., 1935. 'Doppelbrechung, Dichroismus und Feinbau des Aussengliedes der Sehzellen von Frosch.' Z. Zellforsch. mikr. Anat., **22,** 485.
—— 1937. 'Die Doppelbrechung von Karyoplasma, Zytoplasma und Mataplasma.' Protoplasma-Monographien, **11,** Berlin (Börnträger).
ÖSTRAND, F. S., 1953. 'The ultrastructure of the inner segments of the retinal rods of the guinea pig as revealed by electron microscopy.' J. cell. comp. Physiol., **42,** 45.
—— 1956. 'The ultrastructure of cells as revealed by the electron microscope.' Internat. Rev. Cytol., **5,** 455.
EILL, R., 1946. '*Ctenoctophrys chattoni,* n. g., n. sp., infusoire planktonique octoradié à caractère de méduse et de cténophore.' C.r. Acad. Sc., **222,** 683.
ILMER, E. N., 1955. 'The physiology of vision.' Ann. Rev. Physiol., **17,** 339.
OLKEN, J. J., and PALADE, G. E., 1952. 'Fine structure of chloroplasts in two flagellates.' Nature, **170,** 114.
—— —— 1953. 'An electron microscope study of two flagellates. Chloroplast structure and variation.' Ann. New York Acad. Sci., **56,** 873.
—— 1956*a*. 'A molecular morphology of *Euglena gracilis* var. *bacillaris.*' J. Protozool., **3,** 211.
—— 1956*b*. 'Photoreceptor structures. I. Pigment monolayers and molecular weight.' J. cell. comp. Physiol., **48,** 349.

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